IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellants: Lara Madison, Gjalt W. Huisman, and Oliver P. Peoples

Serial No.: 09/235,875 Art Unit: 1638

Filed: January 22, 1999 Examiner: Russell Kallis

For: *TRANSGENIC SYSTEMS FOR THE MANUFACTURE OF POLY(3-HYDROXY-BUTYRATE-CO-3-HYDROXYHEXANOATE)*

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

This is an appeal from the final rejection of claims 1, 6, 7, 10 and 14-21 in the Office Action mailed October 21, 2004, in the above-identified patent application. A Notice of Appeal was filed on January 20, 2005. The Commissioner is hereby authorized to charge $500.00, the fee for the filing of this Appeal Brief for a large entity, to Deposit Account No. 50-3129. Submitted with this Appeal Brief is a Petition for an Extension of Time, to extend the period for response for one-month, to and including April 20, 2005. The Commissioner is hereby authorized to charge $120.00, the fee for a large entity, to Deposit Account No. 50-3129.

It is believed that no additional fee is required with this submission. However, should an additional fee be required, the Commissioner is hereby authorized to charge the fee to Deposit Account No. 50-3129.

(1) REAL PARTIES IN INTEREST

The real party in interest of this application is the assignee, Metabolix, Inc., Cambridge, Massachusetts, which has entered into licensing agreements with other parties.

(2) RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to appellant, the undersigned, or appellant's assignee which directly affects, which would be directly affected by, or which would have a bearing on the Board's decision in this appeal.

(3) STATUS OF CLAIMS

Claims 1, 6, 7, 10 and 14-21 are pending and on appeal. Claims 2-5, 8-9, 11-13, and 22-34 have been cancelled. The text of each claim on appeal, and as pending, is set forth in an Appendix to this Appeal Brief.

(4) STATUS OF AMENDMENTS

The claims were last amended in the Amendment and Response, filed January 20, 2005. An appendix sets forth the claims on appeal.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Known biological systems for the production of polyhydroxyalkanoates (PHAs) containing 3-hydroxyhexanoate (3HH) are inefficient. Bacteria such as *E. coli* do not normally produce PHAs and have not previously been described to produce 3HH copolymers, although they have been engineered to express heterologous genes to produce polyhydroxybutyrate ("PHB") and other polyhydroxyalkanoates. The claimed method is based on the discovery that bacteria can be genetically engineered to produce polyhydroxybutyrate-co-

polyhydroxyhexanoate (PHBH) by selecting a PHA polymerase that accepts six carbon substrates, and then providing the appropriate substrates. The various pathways that can be utilized to produce PHBH are shown in Figures 2-5. The bacteria can be further engineered to utilize one or more of these pathways to provide substrates for the PHA polymerase.

Claim 1 defines a method for the biological production of polyhydroxyalkanoate containing 3-hydroxyhexanoate, which includes providing genetically engineered bacteria expressing a phbA thiolase gene encoding an enzyme that converts butyryl-CoA and acetyl CoA to beta-ketohexanoyl-CoA (page 11, lines 19-24; page 12, lines 22-26; page 13, lines 20-26), a phbB reductase gene that encodes an enzyme that converts beta-ketohexanoyl-CoA to beta-hydroxyhexanoyl-CoA (page 12, lines 25-26; page 21, lines 18-21), and a phbC polymerase gene that encodes an enzyme that polymerizes 3-hydroxybutyryl CoA and-3-hydroxyhexanoyl-CoA (page 21, lines 11-15; Examples 2, 3, and 5), where the enzymes are expressed in a sufficient amount to produce polyhydroxybutyrate-co-polyhydroxyhexanoate. The phbC polymerase gene can be obtained from a bacterial strain such as *Aeromonas caviae*, *Comamonas testosteroni, Thiocapsia pfenigii, Chromatium vinosum, Bacillus cereus, Nocardia carolina, Nocardia salmonicolor, Rhodococcus ruber, Rhodococcus rhodocrous,* or *Rhodospirilum rubrum* (page 3, line 19-25; page 10, line 28 to page 11, line 2; page 12, lines 1-15). The bacteria may also be engineered or selected to express genes encoding a ß-hydroxyacyl-ACP-coenzyme A transferase (page 5, lines 20-25; page 15, lines 18-23) and/or a D-specific enoyl-CoA hydratase (page 5, lines 20-25; page 23, lines 26-29) (See Figure 5). The bacteria may also express one or more fatty acid biosynthetic enzymes, such as enzymes which convert acyl ACP to acyl CoA,

including ACP-CoA transacylase, acyl ACP thioesterase, or acyl CoA synthase (page 15, lines 8-20; Example 6; Figures 5 and 10).

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues presented on appeal are:

(1) whether claims 1, 6, 7, 10, and 14-21 comply with the written description requirement as required by 35 U.S.C. § 112, first paragraph; and

(2) whether claims 1, 6, 7, 10, and 14-21 are enabled as required by 35 U.S.C. § 112, first paragraph.

(7) GROUPING OF CLAIMS

The claims do not stand or fall together as discussed below.

(8) ARGUMENTS

(a) The Claimed Invention

Polyhydroxyalkanoates (PHAs) are natural, thermoplastic polyesters and can be processed by traditional polymer techniques for use in an enormous variety of applications, including consumer packaging, disposable diaper linings and garbage bags, food and medical products. Several factors are critical for economic biological production of PHAs, including substrate costs, fermentation time, and efficiency of downstream processing.

As described above, the production of PHAs containing 3-hydroxy-co-hydroxyhexanoate (3H-co-HH) in known biological systems is inefficient. However, the Appellants have discovered that microorganisms, such as *E. coli*, which do not normally produce PHAs, can be genetically engineered to produce PHAs by the introduction of a PHA synthase gene which

encodes an enzyme that accepts C6 substrates and optionally, additional transgenes, such as genes encoding ß-ketothiolase, acetoacetyl-CoA reductase, ß-ketoacyl-CoA reductase, enoyl-CoA hydratase and/or ß-hydroxyacyl-ACP-coenzymeA transferase. The genes are preferably selected on the basis of the substrate specificity of their encoded enzymes for the production of the 3HH polymers as wekk as PHB.

The Appellants have also discovered that biological systems for the production of PHAs containing 3-hydroxy-co-hydroxyhexanoate (3H-co-HH) can be improved by using transgenic organisms with faster growth rates and/or by genetically engineering these organisms to produce the co-monomer 3-hydroxyhexanoic acid from cheaper feedstocks, such as butyrate or butanol, or directly from glucose by incorporating genes encoding enzymes which can channel cellular intermediates to butyryl-CoA, thereby improving the economics of PHA production using transgenic organisms. Enzyme activities desirable for conversion of metabolic intermediates into *R*-3-hydroxyhexanoyl CoA, include butyryl CoA dehydrogenase activity and acyl CoA:ACP transferase activities. The latter conversion is catalyzed either by a single protein or by a combination of thioesterase and acyl CoA synthase activities. The flux of normal cellular metabolites to 3-hydroxyhexanoate is redirected via one or more of three different pathways. These three pathways generate 3-hydroxyhexanoate, either (1) using a butyrate fermentation pathway, for example using enzymes from *Clostridium acetobutylicium* (Figure 3), (2) using fatty acid biosynthetic enzymes, for example from *E. coli* (Figure 5), or (3) using a fatty acid oxidation complex, for example, from *Pseudomonas putida* (from Figure 6). In a preferred embodiment, *E. coli* is engineered to synthesize PHBH from either inexpensive carbohydrate

feedstocks such as glucose, sucrose, xylose and lactose or mixtures of such carbohydrates and fatty acids as the only carbon source by introducing genes encoding enzymes that convert cellular metabolites to 3-hydroxyhexanoyl CoA. It is crucial that the expression of all the genes involved in the pathway be adequate for efficient PHA synthesis in recombinant *E. coli* strains.

An example of a biosynthetic pathway that results in *R*-3-hydroxyhexanoyl CoA formation involves the elongation of butyryl CoA to 3-ketohexanoyl CoA which can subsequently be reduced to the monomer precursor, as shown in Figure 4. Butyryl CoA is formed by butyrate fermenting organisms such as *C. acetobutylicum* in a four step pathway from acetyl CoA. Elongation of butyryl CoA to 3-ketohexanoyl CoA is catalyzed by a thiolase. The complete pathway thus involves (1) the PHB biosynthetic thiolase, (2) the three enzymes from *C. acetobutylicum* that form butyryl CoA, (3) a second thiolase, specific for 3-ketohexanoyl CoA, (4) a reductase specific for this substrate, and (5) a PHB polymerase that accepts both 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl CoA.

In *P. putida*, monomers for PHA biosynthesis are derived from the fatty acid oxidation pathway when alkanes or oxidized alkanes are provided as carbon and energy source. The intermediate in this pathway that is channeled to PHA biosynthesis is *S*-3-hydroxyacyl CoA (preferentially C_8 and C_{10}) which undergoes epimerization by the FaoAB complex to the *R*-isomer. The combined action of epimerase and PHA polymerase provides C_6 to C_{14} monomers for PHA. Consequently, a combination of this epimerase and a 3-hydroxyhexanoyl CoA accepting PHA polymerase provides the biosynthetic capability to synthesize PHBH from fatty acids in transgenic organisms, as shown by Figure 5. Mixtures of fatty acids and carbohydrates

that are useful feedstocks for fermentative production as the 3HB monomer can be derived from acetyl CoA, where the 3HH component is from fatty acids.

P. putida and *P. aeruginosa* synthesize PHAs composed of medium-chain length 3-hydroxy fatty acids when grown on sugars. The predominant monomer in these PHAs is 3-hydoxydecanoate. A similar pathway can be engineered for the synthesis of PHBH in recombinant microorganisms such as *E. coli, R. Eutropha* and *P. putida*, as shown by Figure 6. Besides a polymerase that accepts the 3-hydoxybutyryl CoA and 3-hydroxyhexanoyl CoA precursors, an enzymatic activity that converts 3-hydroxyacyl ACP into 3-hydroxyacyl CoA or 3-ketoacyl ACP into 3-ketoacyl CoA is required. Deregulation of fatty acid biosynthesis and increased activity of this pathway subsequently provides the substrate for PHBH formation. The critical enzymatic activity in this pathway is the conversion of the 3-hydroxyacyl ACP to the CoA derivative. Thioesterases and acyl CoA synthases can accomplish this step. Alternatively, acyl ACP:CoA transferase can be used to facilitate this step in the PHA pathway.

(b) Rejections Under 35 U.S.C. § 112, first paragraph

i. Rejection of Claims 1, 6, 7, 10, 14-21 under 35 U.S.C. § 112, first paragraph (written description)

The Legal Standard

Both the written description and enablement requirements are defined by 35 U.S.C. § 112, first paragraph, which states that the patent specification must contain "a written description of the invention, and of the manner and process of making and using it...[such] as to enable any person of ordinary skill in the art to which it pertains ... to make and use the same ... " The

purpose of the written description requirement is to prevent a patentee from later asserting that he invented something which he did not. Thus the patentee must "recount his invention in such detail that his future claims can be determined to be encompassed within his original creation." *Vas- Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1561, 19 U.S.P.Q.2d 1111, 1115 (Fed. Cit 1991).

For many years the leading case for the written description requirement in the biotechnology and pharmaceutical arts was *Eli Lilly v. Univ. of Calif. Board of Regents*, in *Regents of University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 U.S.P.Q.2d 1398 (Fed. Cir. 1997), *cert denied*, 523 U.S. 1089 (1998). The Federal Circuit evaluated whether claims to recombinant production of human insulin in U.S. Patent No. 4,652,525 met the written description requirement. The court determined that the specification failed to comply with the written description requirement for only disclosing a single species of DNA encoding non-human insulin.

The Federal Circuit has since held that that the written description requirement can be met by a functional description of claimed materials, if coupled with a known or disclosed correlation between function and structure. *Enzo Biochem, Inc., v. Gen-Probe, Inc.*, 296 F.3d 1316, 63 U.S.P.Q.2d 1609 (Fed. Cir.2002) ("*Enzo II*"). The court held that a patentee complied with the written description requirement by depositing biological material in a public depository. In *Enzo II*, the Federal Circuit rejected its narrow interpretation of *Eli Lilly* that the disclosure of the sequence was always necessary, and instead adopted a broader interpretation of the types of disclosures that comply with the written description requirement. The court adopted provisions from the Guidelines issued by the U.S. Patent and Trademark Office that state that the written

description requirement can be met by a functional description of claimed materials, if coupled with a known or disclosed correlation between function and structure. The court found that the written description requirement was met when, in the knowledge of the art, the disclosed function is sufficiently correlated to a particular, known structure. This standard was subsequently affirmed and clarifed in the decision of *Amgen Inc. v. Hoechst Marion Roussel, Inc. and Transkaryotic Therapies, Inc.* 314 F.3d 1313, 65 USPQ 2d (Fed. Cir. 2003).

Claims 1, 6, 7, 10, and 14-21 Satisfy the Written Description Requirement

With regard to the NEW MATTER rejection, the Examiner alleges that there is no support in claim 1 for "a *phbC* polymerase gene that encodes an enzyme that polymerizes 3-hydroxybutyrl CoA and 3-hydroxyhexanoyl-CoA". However, page 21, lines 11-15, discloses that **3-hydroxyhexanoyl CoA accepting** PHA polymerase genes can be obtained from *A. caviae, C. testosteroni, T. pfenigii,* and possibly *P. denitrificans* and *S. natans*.

In addition, support can also be found in Example 3, which describes the production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH) by construction of transgenic *E. coli* strains that express chromosomally encoded phaC from *N. salmonicolor*. Note that the only polymerase that is provided is the one obtained from *N. salmonicolor*, which acts on both substrates. Furthermore, Examples 2 and 5 disclose that PHBH was produced from glucose and butyrate in E. coli expressing a plasmid (pMBXc12J12) containing the PHB polymerase from *A. caviae*, which confirms that this enzyme accepts **both** 3-hydroxybutyrl CoA and 3-hydroxyhexanoyl-CoA. Construction of the plasmid and the **sequence** for the *A. caviae* polymerase gene is disclosed in the publication cited in Example 2 on page 21, lines 24-26

(Fukui & Doi. *J. Bacteriol.* 179:4821-4830 (1997) (submitted with IDS; copy enclosed in the Appendix)). Finally, Figure 9 is a schematic of selection for a PHBH recombinant pathway in *E. coli* using the PHA polymerase gene phaC from *P. putida*. The schematic clearly shows that the polymerase acts on 3-hydroxyhexanoyl-CoA. Accordingly, there is ample support in the specification for the amendments to the claim and it is clear that the Appellants were in possession of the broad substrate polymerase at the time of filing.

With regard to the other claim rejections under 35 U.S.C. § 112, first paragraph (written description), please consider the following comments.

Generally, there is an inverse correlation between the level of skill and knowledge in the art and the specificity of disclosure necessary to satisfy the written description requirement. Information which is well known in the art need not be described in detail in the specification. See e.g. *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379-80, 231 USPQ 81, 90 (Fed. Cir. 1986). Appellants are claiming a method for the production of a polymer in bacteria that incorporates **new combinations** of genes and enzymes with **known** sequences.

The enzymes set forth in the claims are a phbA thiolase gene, a phbB reductase gene, a phbC polymerase gene that encodes an enzyme that polymerizes 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA (claims 1, 6, 7, 14 and 15), a gene encoding a ß-hydroxyacyl-ACP-coenzyme A transferase (claim 10), a gene encoding a D-specific enoyl-CoA hydratase (claim 16), three enzymes from *C. acetobutylicum* that form butyryl CoA, a thiolase specific for 3-ketohexanoyl CoA, a reductase specific for 3-ketohexanoyl CoA (claim 17), and fatty acid biosynthetic enzymes including ACP-CoA transacylase, acyl ACP thioesterase, and acyl CoA

synthase (claims 18-21). Each of these genes and enzymes were well known to those skilled in the art, commercially available and sufficiently identified in the specification as of the date of filing to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention.

For example, sequence information can be obtained from the cited publications, such as those on page 1, line 29 to page 2, lines 12, and actual DNA can be obtained from the authors of the cited publications or purchased from commercial suppliers, such as the American Type Culture Collection (ATCC). Published amino acid and nucleotide sequence listings for the various genes can also be obtained from GenBank or the National Center for Biotechnology Information (NCBI), as demonstrated by the Appellants in their response and amendment mailed on March 10, 2003 (copy enclosed in the Appendix), in which published sequence listings from the NCBI were submitted. Included were *phb*B amino acid and nucleotide sequences from *Asospirillum brasilense* and *Pseudomonas putida*; *phb*A amino acid and nucleotide sequences from *Pseudomonas putida*; and *phb*C amino acid and nucleotide sequences from *Pseudomonas putida*, *Rhodobacter sphaeroides*, *Azorhizobium caulinodans*, *A. eutrophus*, and *Pseudomonas sp.* In addition, in their response filed August 5, 2004, the Appellants submitted copies of the results of searches of GenBank for *acyl CoA synthase*, *acyl ACP thioesterase,* and *ACP-CoA transacylase* sequences published before the priority date of the application, January 22, 1998 (copy enclosed in the Appendix).

Furthermore, one of ordinary skill would know that each of the genes and enzymes used in the claimed methods can be easily isolated and sequenced using methods known in the art or

described in the specification. As noted above, on page 1, line 29 to page 2, line 11, the specification discloses a number of publications, which describe methods for producing PHAs in natural or genetically engineered organisms and makes special reference to patents that disclose the genes encoding the reductase, thiolase, and PHB polymerase of claims 1, 6, 7, 14, and 15 (U.S. Patent Nos. 5,245,023; 5,250,430; 5,480,794; 5,512,669; 5,534,432 to Peoples and Sinskey; page 2, lines 2-5). In addition, on page 10, line 29 to page 11, line 2, the specification states that useful PHA synthase genes have been isolated from, for example, *Aeromonas caviae* (Fukui & Doi, *J. Bacteriol.* 179: 4821-30 (1997)), *Rhodospirillum rubrum* (U.S. Patent No. 5,849,894), *Rhodococcus ruber* (Pieper & Steinbuechel, *FEMS Microbiol.Lett.* 96(1): 73-80 (1992)), and *Nocardia corallina* (Hall et. al., *Can. J. Microbiol.* 44: 687-91 (1998)). All of these references were submitted with the Information Disclosure Statement. Furthermore, the Fukui & Doi and Hall references disclose that the PHA synthase genes from *A. caviae* (Fukui & Doi, page 4828, 1st full paragraph) and *Nocardia corallina* (Hall et al., pages 690-691) can polymerize 3-hydroxybutyrl CoA and 3-hydroxyhexanoyl-CoA. Therefore, it clear that there is sufficient support for these enzymes in the specification and in the literature, especially the phbC genes from the bacteria recited in claims 7 and 15.

The ß-hydroxyacyl-ACP-coenzyme A transferase gene, cited in claim 10, encodes an enzyme that converts 3-hydroxyacyl ACP to the CoA derivative. This step in the polyhydroxyalkanoate pathway is facilitated by acyl ACP:CoA transferase activity. The specification states that genes that encode this enzyme can easily be identified in bacteria that produce polyhydroxyalkanoates from oxidized carbon sources, such as carbohydrates (see page

15, lines 18-23, of the specification). In addition, the identification of genes encoding enzymes that convert acyl ACP to acyl CoA is presented in Figure 10 as a screen that makes use of the very user friendly *lux* system of *Vibrio fischeri*. One merely assays for light generation that results from the induction of the transgenic lux system. Such light generation implicates ACP::CoA transferase activity that is present in the system.

On page 24, lines 4-9, the specification recites a specific reference relating to the *phaJ* gene encoding an enoyl-CoA hydratase, cited in claim 16 (Fukui and Doi, *J. Bacteriol.* 179: 4821-30 (1997) (submitted with IDS; copy enclosed in the Appendix), and describes how to isolate this gene from chromosomal DNA prepared from *A. caviae* strain FA-440, obtained from the Japanese Culture Collection under accession number FERM BP 3432 (U.S. Patent No. 5,292,860)).

On page 12, line 18 to page 13, line 19, the specification describes a thiolase specific for 3-ketohexanoyl, a reductase specific for 3-ketohexanoyl, and enzymes from *C. acetobutylicum* (*hbd*, *crt*, *bdh*) that form butyryl CoA, which are cited in claim 17. With regard to the enzymes from *C. acetobutylicum*, a number of publications are recited, such as Boynton et al. *J. Bacteriol.* 178(11): 3015-3024 (1996) (submitted with IDS; copy enclosed in the Appendix), which describe the isolation of these genes. In addition, Example 3 describes the isolation of the genes using PCR. The specification also makes reference to GenBank (page 13, line 4), demonstrating that the sequences of these genes may be accessed through a public depository. In addition, on page 11, lines 10-18, the specification recites Ploux *et al.* (1988) and Haywood et al.(1988), which disclose that 3-ketohexanoyl CoA is a substrate for reductase enzymes from *Z. ramigera*

and *R. eutropha*. The specification also recites Haywood *et al.* (1988) on page 11, lines 19-24, which discloses that *R. eutropha* has two 3-ketothiolases, one of which has activity for higher 3-ketoacyl CoA's (i.e. 3-ketohexanoyl CoA).

Finally, the fatty acid biosynthetic enzymes in claims 18-21 are defined by their substrates. Many are known, cloned and well characterized. For example, see the enclosed copies of the results of searches of GenBank for *acyl CoA synthase*, *acyl ACP thioesterase*, and *ACP-CoA transacylase* that were submitted by the Appellants in their response dated August 5, 2004. Also see Pramanik et al. 137(1): 469-473 (1979) (submitted with IDS; copy enclosed in the Appendix), cited at page 14, line 20, and Bouqin et al. *Mol. Gen. Genet.* 246(5): 628-637 (1995) (submitted with IDS; copy enclosed in the Appendix), cited at page 25, line 26. Homologous genes are readily isolated from bacteria such as *R. eutropha*, *A. latis*, *C. testosteroni*, *P. denitrificans*, *R. ruber*, and other PHA and non-PHA producers using the same methods to identify the *fao*AB (fatty acid oxidation) genes in *P. putida* KT2442. This is explicitly stated at lines 30-3, bridging pages 14 and 15 of the specification. Furthermore, epimerase activity had been detected in the fatty acid oxidation complexes of *E. coli* and *P. fragi*. As disclosed at page 14, lines 21-26, each of the *Fao*AB complex subunits were cloned and expressed to show the substrate specificity of components of the PHA pathway in *P. putida*.

ii. Rejection of Claims 1, 6, 7, 10, 14-21 under 35 U.S.C. § 112, first paragraph (enablement)

The Legal Standard

The Court of Appeals for the Federal Circuit (CAFC) has described the legal standard for enablement under § 112, first paragraph, as whether one skilled in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art, without undue experimentation (*See, e.g.*, *Genentech, Inc. v. Novo Nordisk A/S*, 108 F3d at 165, 42 USPQ2d at 1004 (quoting *In re Wright*, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993); See also *In re Fisher*, 427 F.2d at 839, 166 USPQ at 24; *United States v. Telectronics, Inc.*, 857 F.2d 778 (Fed. Cir. 1988); *In re Stephens,* 529 F.2d 1343 (CCPA 1976)). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation (*M.I.T. v. A.B. Fortia*, 774 F.2d 1104 (Fed. Cir. 1985)). In addition, as affirmed by the Court in *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524 (Fed. Cir. 1987), a patent need not teach, and preferably omits, what is well known in the art.

Whether the disclosure is enabling is a legal conclusion based upon several underlying factual inquiries. See *In re Wands*, 858 F.2d 731, 735, 736-737, 8 USPQ2d 1400, 1402, 1404 (Fed. Cir. 1988). A determination of undue experimentation is a conclusion based on weighing many factors, not just a single factor. Many of these factors have been summarized in *In re Forman,* 230 USPQ 546, 547 (Bd. Pat. App. & Int. 1986) and set forth in *In re Wands*. They are: (1) The quantity of experimentation necessary (time and expense); (2) The amount of direction

or guidance presented; (3) The presence or absence of working examples of the invention; (4) The nature of the invention; (5) The state of the prior art; (6) The relative skill of those in the art; (7) The predictability or unpredictability of the art; and (8) The breadth of the claims. The M.P.E.P. explains that "[i]t is improper to conclude that a disclosure is not enabling based on an analysis of only one of the above factors while ignoring one or more of the others." Thus, a conclusion of nonenablement must be based on the evidence as a whole, as related to each of these factors (see M.P.E.P. § 2164.01 (a)).

In cases that involve unpredictable factors, "the scope of the enablement obviously varies inversely with the degree of unpredictability of the factors involved." *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation 'must not be unduly extensive.' *Atlas Powder Co., v. E.I. DuPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984). There is no requirement for examples. *In re Borkowski, 422 F.2d 904, 57 C.C.P.A. 946, 1970.*

In its most recent decision regarding issues of written description and enablement as defined by 35 U.S.C. § 112, *Amgen, Inc. v. Hoechst Marion Roussel, Inc. and Transkaryotic Therapies, Inc.*, 314 F.3d 1313 (Fed. Cir. 2003), the Court of Appeals for the Federal Circuit continued in the manner of *Enzo II* and applied a broad interpretation of the types of disclosures that comply with the written description requirement. Similarly, in *Amgen*, the Federal Circuit adopted a broad interpretation of the types of disclosures that meet the enablement requirement.

The Federal Circuit stated "because the claim terms at issue here are not new or unknown biological materials that ordinarily skilled artisans would easily miscomprehend. Instead, the claims of Amgen's patents refer to types of cells that can be used to produce recombinant human EPO. [...] This difference alone distinguishes *Eli Lilly*, because when used, as here, merely to identify types of cells (instead of undescribed, previously unknown DNA sequences), the words 'vertebrate' and 'mammalian' readily 'convey[] distinguishing information concerning [their] identity' such that one of ordinary skill in the art could 'visualize or recognize the identity of members of the genus." *Amgen,* 314 F.3d at 1332, citing *Eli Lilly*, 119 F.3d at 1567, 1568, 43 U.S.P.Q.2d at 1406.

The Federal Circuit stated that Amgen's invention was not "the location of the control sequences and EPO DNA in relation to the cell, but rather the production of human EPO using those sequences." *Id.* The court held that the claims were enabled based on two rationales. First, in response to TKT's argument that the claims were not enabled since the specification did not teach a method for making the EPO using endogenous DNA, the court explained, "The specification's failure to disclose the later-developed endogenous activation technology cannot invalidate the patent." *Id.* at 1335, citing *Amgen*, 126 F. Supp. 2d at 160, 57 U.S.P.Q.2d at 516. Second, the court referred to earlier cases for the rule that "the law makes clear that the specification need teach only one mode of making and using a claimed composition." *Id.*, citing *Johns Hopkins Univ. v. Cellpro, Inc.*, 152 F.3d 1342, 1361, 47 U.S.P.Q.2d 1705, 1719 (Fed. Cir. 1998); *Engel Indus. Inc. v. Lockformer Co.*, 946 F.2d 1528, 1533, 20 U.S.P.Q.2d 1300, 1304 (Fed. Cir. 1991). The court also held that the '422 patent was enabled since the specification

"described and enabled at least one way of obtaining EPO purified from mammalian cells in culture." *Id.*

A proper analysis of the *Wands* factors shows that the claims satisfy the enablement requirement. It clear from the amount of direction or guidance presented in the specification, the presence of working examples, the state of the prior art, and the relative skill in the art that one of ordinary skill in the art would be able to make and use the claimed transgenic bacteria for the production of polyhydroxybutyrate-co-polyhydroxyhexanoate, without **undue experimentation.**

The issue is whether or not one skilled in the art would know what genes are required to make the claimed bacteria, and whether or not it would require undue experimentation to make and use the claimed bacteria. As demonstrated by the disclosure and actual working examples in the specification, those skilled in the art would know what enzymes are required and would either be able to use those publicly available, described in the literature, or obtained without undue experimentation using the information provided by Appellants. Unlike in some systems, such as eukaryotic cells, genes have been identified by their activities and transferred into bacteria, either in plasmids or incorporated into the genome, without the sequence being known, for decades. What is important is to know a source for the genes, and what the genes must encode.

As is apparent from the discussion of the recent Federal Circuit decisions, this requirement may be different if one is claiming the genes, but in this application, appellants are claiming methods of making a polymer using new combinations of known materials, very similar to the fact situation in *Amgen v. Hoescht, et al.*, 314 F.3d 1313 (Fed. Cir. 2003).

The enzymes set forth in the claims are a phbA thiolase gene, a phbB reductase gene, a phbC polymerase gene that encodes an enzyme that polymerizes 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA (claims 1, 6, 7, 14 and 15), a gene encoding a ß-hydroxyacyl-ACP-coenzyme A transferase (claim 10), a gene encoding a D-specific enoyl-CoA hydratase (claim 16), three enzymes from *C. acetobutylicum* that form butyryl CoA, a thiolase specific for 3-ketohexanoyl CoA, a reductase specific for 3-ketohexanoyl CoA (claim 17), and fatty acid biosynthetic enzymes including ACP-CoA transacylase, acyl ACP thioesterase, and acyl CoA synthase (claims 18-21).

The specification clearly discloses how to obtain the genes and enzymes that are used in the methods and recites specific publications, which describe these materials in detail. For example, on page 1, line 29 to page 2, line 11, the specification discloses a number of publications, which describe methods for producing PHAs in natural or genetically engineered organisms and cites references which also disclose and claim the genes encoding reductase, thiolase, and PHB polymerase (claims 1, 6, 7, 14, and 15). In addition, on page 10, line 29 to page 11, line 2, the specification discloses a number of organisms from which useful PHA synthase genes have been isolated. There is also adequate support in the specification for all of the claimed genes and enzymes, including a phbC polymerase gene encoding an enzyme that polymerizes 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl CoA (page 21, lines 11-15 and Examples 2, 3 and 5). Furthermore, the genes and enzymes may be isolated using methods commonly known in the art or described in the publications, obtained from the authors of the

cited publications, or purchased from commercial suppliers, such as the American Type Culture Collection (ATCC).

Once a gene is identified, it is routine in the art to incorporate the gene into a plasmid for transfection of bacteria. PCR methods are well within the skill of one in the art and would not require undue experimentation. There also is sufficient direction and guidance given by the specification to construct plasmids and express the claimed genes in bacteria, such as *E. coli* (claim 14) (see page 18, lines 15-28 and Examples). Furthermore, the experimental protocols are routine in the art and expression vectors, restriction enzymes and ligation enzymes are also commercially available.

Although there is no requirement for examples, Appellants have provided numerous working examples which not only demonstrate that one can use the claimed enzymes to produce HH containing copolymers, but that one can isolate the desired enzymes with only routine experimentation. Example 1, on page 19 of the specification, discusses a routine method used for the isolation of specific genes. This Example illustrates the use of PCR to amplify and isolate the *pha*C gene encoding the polyhydroxyalkanoate ("PHA") polymerase enzyme from *N. salmonicolor* chromosomal DNA. Example 2 discloses the construction of plasmids containing the *A. caviae* PHB polymerase gene (pMBXc12J12) and *R. eutropha* phbAB genes (pSU18-AB1) and cites to two publications, which describe the genes (page 21, lines 24-29). Example 3 further shows, using the same methods of Example 1, how one would isolate the *hbd*, *crt*, and *bdh* genes from *C. acetobutylicum* (claim 17) (see page 22 of the specification).

Example 4, at pages 23 and 24 of the specification, clearly illustrates a method of isolating the *pha*J gene encoding an enoyl-CoA hydratase (claim 16) from chromosomal DNA prepared from an *A. caviae* strain. Specific primers were used in polymerase chain reactions to amplify the gene. The sequence of the primers are shown on page 24, lines 9-15. Plasmids were constructed and subsequently used to transform recipient *E. coli* cells. Once transformed, the cells express the hydratase gene as evidenced by the production of polyhydroxybutyrate-co-polyhydroxyhexanoate containing 2.6% HH comonomer. This copolymer was analyzed *via* gas chromatography as shown at page 24, lines 24-25.

The enzymes are defined by their substrate specificity. As discussed at page 5, lines 25-27, of the specification, "the genes are preferably selected on the basis of the substrate specificity of their encoded enzymes being beneficial for the production of the 3HH polymers." The substrate, in the presence of its cognate active enzyme, will be readily converted into product (i.e. the substrate for another enzyme). Based upon the specification, one of ordinary skill in the art will appreciate that the presence, or production, of end-product (i.e. polyhydroxybutyrate-co-polyhydroxyhexanoate) is easily measured and characterized using methods well known in the art. Page 18, line 29, to page 19, line 10, describes in detail the method of using gas chromatography to analyze the polyhydroxyalkanoate produced from the claimed methods. Gas chromatography was used in each of Examples 2, 4, and 5 to analyze the final content of the copolymer produced (1% HH comonomer, 2.6% HH comonomer, and 1.2% HH comonomer, respectively).

The ease in which one may assay for the final copolymer product, as described in the preceding paragraph, supports enablement for use of the specific enzymes. The presence of the copolymer end product will tell the investigator whether or not the overall process of providing proper substrate for enzyme catalysis at each step in the copolymer synthetic pathway is successful. The genes encoding each of these enzymes can be isolated by using common PCR techniques discussed above, or obtained from the ATCC, or through catalogs or as described in the literature. All of the claimed enzymes are known and characterized.

The fatty acid biosynthetic enzymes of claims 18-21 make use of distinctly different substrates than those of the other enzymes. The fatty acid biosynthesis pathway requires an enzyme activity that converts acyl ACP into acyl CoA, a reaction catalyzed by an ACP/CoA transacylase or by the combined action of an acyl ACP thioesterase and acyl CoA synthase. It is clear from the specification at page 25, line 20, to page 26, line 12, that the *lux* system of *V. fischeri* is an ideal tool for easily screening genetic sequences encoding the desired activity that converts acyl ACP into acyl CoA. Such a screening tool enables the identification of ACP/CoA transacylase, acyl ACP thioesterase, and acyl CoA synthase enzymatic activity. Once the activity is identified, the corresponding genetic sequence is easily isolated from the plasmid initially used to transform *V. fischeri*. The sequence is subsequently amplified using well known PCR techniques.

Finally, the Appellants have patents on engineering of bacteria and plants to express PHA synthesis genes that issued on patent applications filed more than fifteen years ago. The massive amount of prior art clearly demonstrates that the field is not unpredictable, and that once one

identifies the enzymes to be used, based on their known substrates and known reaction products, it becomes routine to express the genes encoding those enzymes.

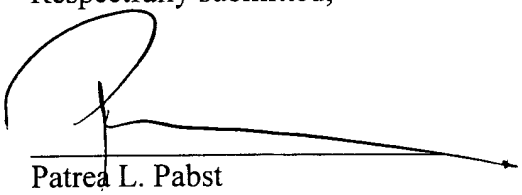
(9) SUMMARY AND CONCLUSION

The rejection under 35 U.S.C. § 112, first paragraph (written description) is improper because the specification sufficiently describes the subject matter in the claims.

The rejection under 35 U.S.C. § 112, first paragraph (enablement) is also improper because it is clear from the direction or guidance given by the specification, the presence of working examples, the state of the prior art, and the relative skill of those in the art that one of ordinary skill in the art could make and use the claimed transgenic bacteria for the production of polyhydroxybutyrate-co-polyhydroxyhexanoate, without undue experimentation.
The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir.1988).

For the foregoing reasons, Appellant submits that claims 1, 6, 7, 10 and 14-21 are patentable.

Respectfully submitted,



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Claims Appendix: Claims On Appeal

1. (previously presented) A method for the biological production of polyhydroxyalkanoate containing 3-hydroxyhexanoate comprising providing genetically engineered bacteria expressing a phbA thiolase gene encoding an enzyme that converts butyryl-CoA and acetyl CoA to beta-ketohexanoyl-CoA, a phbB reductase gene that encodes an enzyme that converts beta-ketohexanoyl-CoA to beta-hydroxyhexanoyl-CoA, and a phbC polymerase gene that encodes an enzyme that polymerizes 3-hydroxybutyryl CoA and-3-hydroxyhexanoyl-CoA, wherein the enzymes are expressed in a sufficient amount to produce polyhydroxybutyrate-co-polyhydroxyhexanoate.

Claims 2-5 (Canceled).

6. (previously presented) The method of claim 1 wherein the phbC polymerase gene is incorporated into the bacterial chromosome.

7. (previously presented) The method of claim 1 for producing a copolymer of polyhydroxyhexanoate comprising providing a phbC polymerase gene from a bacteria selected from the group consisting of *Aeromonas caviae*, *Comamonas testosteroni, Thiocapsia pfenigii, Chromatium vinosum, Bacillus cereus, Nocardia carolina, Nocardia salmonicolor, Rhodococcus ruber, Rhodococcus rhodocrous*, and *Rhodospirilum rubrum.*

Claims 8 and 9 (Canceled).

10. (previously presented) The method of claim 1 wherein the bacteria further comprises a gene encoding ß-hydroxyacyl-ACP-coenzyme A transferase.

Claims 11-13 (Canceled).

14. (previously presented) The method of claim 1 wherein the bacteria is *E. coli.*

15. (previously presented) The method of claim 7 further comprising providing a gene encoding a polymerase from a bacterium selected from the group consisting of *R. eutropha, Klebsiella aerogenes,* and *P. putida.*

16. (previously presented) The method of claim 1 wherein the bacteria expresses a gene encoding a D-specific enoyl-CoA hydratase.

17. (previously presented) The method of claim 1 wherein the bacteria expresses a PHB biosynthetic thiolase, three enzymes from *C. acetobutylicum* that form butyryl CoA, thiolase specific for 3-ketohexanoyl CoA, reductase specific for 3-ketohexanoyl CoA, and PHB polymerase that accepts both 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl CoA.

18. (previously presented) The method of claim 1 wherein the bacteria expresses one or more fatty acid biosynthetic enzymes.

19. (previously presented) The method of claim 18 wherein the fatty acid biosythetic enzymes convert acyl ACP to acyl CoA.

20. (original) The method of claim 19 where the enzymes are selected from the group consisting of ACP-CoA transacylase, acyl ACP thioesterase, and acyl CoA synthase.

21. (original) The method of claim 20 wherein the enzymes are acyl ACP thioesterase and acyl CoA synthase.

Claims 22- 34 (canceled).

TABLE OF CONTENTS

Appendix: References cited in Appeal Brief

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Revised: July 5, 2002.

1: AF098459. Rhodobacter sphae...[gi:27573537] Links

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VERSION     AF098459.2  GI:27573537
KEYWORDS    .
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REFERENCE   1  (bases 1 to 2829)
  AUTHORS   Kim,J.-H. and Lee,J.K.
  TITLE     Cloning, nucleotide sequence and expression of gene coding for
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  JOURNAL   J. Microbiol. Biotechnol. 7, 229-236 (1997)
REFERENCE   2  (bases 1 to 2829)
  AUTHORS   Kim,J.-H. and Lee,J.K.
  TITLE     Direct Submission
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            Shin-su Number 1, Seoul 121-742, Korea
REFERENCE   3  (bases 1 to 2829)
  AUTHORS   Kim,J.-H. and Lee,J.K.
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1: AJ006237. Azorhizobium caul...[gi:3152943]

Links

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OCUS        ACA6237                 1752 bp    DNA     linear   BCT 07-OCT-1998
EFINITION   Azorhizobium caulinodans phbC gene.
CCESSION    AJ006237
ERSION      AJ006237.1  GI:3152943
EYWORDS     PHB synthase; phbC gene.
OURCE       Azorhizobium caulinodans
  ORGANISM  Azorhizobium caulinodans
            Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
            Hyphomicrobiaceae; Azorhizobium.
EFERENCE    1
  AUTHORS   Mandon,K., Michel-Reydellet,N., Encarnacion,S., Kaminski,P.A.,
            Leija,A., Cevallos,M.A., Elmerich,C. and Mora,J.
  TITLE     Poly-beta-hydroxybutyrate turnover in Azorhizobium caulinodans is
            required for growth and affects nifA expression
  JOURNAL   J. Bacteriol. 180 (19), 5070-5076 (1998)
  MEDLINE   98422458
   PUBMED   9748438
EFERENCE    2  (bases 1 to 1752)
  AUTHORS   Michel-Reydellet,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-MAY-1998) Michel-Reydellet N., Biotechnologie,
            Institut Pasteur, 25 rue du Dr Roux, Paris, 75724, FRANCE
EATURES             Location/Qualifiers
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     CDS            1..1752
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ASE COUNT 343 a 559 c 556 g 294 t

RIGIN

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'/

Revised: July 5, 2002.

Mar 6 2003 11:06:01

1: Z80158. Pseudomonas sp. p...[gi:3115089]

Links

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LOCUS       ALPHAC                   275 bp    DNA     linear   BCT 12-MAY-1998
DEFINITION  Pseudomonas sp. phaC genc.
ACCESSION   Z80158
VERSION     Z80158.1  GI:3115089
KEYWORDS    phaC gene; polyhydroxybutyratc synthase.
SOURCE      Pseudomonas sp.
  ORGANISM  Pseudomonas sp.
            Bacteria; Proteobacteria.
REFERENCE   1
  AUTHORS   Ja Shin,K., Youn Sung,K., Seung Goun,L., Won Jung,C., Seok Youn,K.,
            Ook Joon,Y., Ghun Bin,Y. and Jang Ryol,L.
  TITLE     Cloning of Alkaligenes lactus Poly-b-hydroxyalkanoic Acid
            Biosynthetic Genes and Their Expression in Escherichia coli
  JOURNAL   Unpublished
  REMARK    (sites)
REFERENCE   2  (bases 1 to 275)
  AUTHORS   Youn Sung,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-SEP-1996) Youn Sung K., Korea Research Institute of
            Bioscience and Biotechnology, Plant Cell and Molecular Biology
            Researt, Yusung, Taejon, Korea
FEATURES             Location/Qualifiers
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1: J05003. A.eutrophus poly-...[gi:141958]

Links

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OCUS       AFAPHBAA                 2768 bp    DNA     linear   BCT 26-APR-1993
EFINITION  A.eutrophus poly-beta-hydroxybuterate-C (phbC) genc, complete cds
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CCESSION   J05003
ERSION     J05003.1  GI:141958
EYWORDS    poly-beta-hydroxybuterase polymcrasc.
OURCE      Ralstonia eutropha
 ORGANISM  Ralstonia eutropha
           Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
           Ralstoniaceae; Ralstonia.
EFERENCE   1  (bases 1 to 2768)
 AUTHORS   Peoples,O.P. and Sinskey,A.J.
 TITLE     Poly-beta-hydroxybutyratc (PHB) biosynthesis in Alcaligenes
           eutrophus H16. Identification and characterization of the PHB
           polymerase gene (phbC)
 JOURNAL   J. Biol. Chem. 264 (26), 15298-15303 (1989)
 MEDLINE   89359357
  PUBMED   2670936
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Items 1-3 of 3 One page.

☐ **1:** U32701 Links

Haemophilus influenzae Rd KW20 section 16 of 163 of the complete genome
gi|3212182|gb|U32701.1|[3212182]

☐ **2:** U91631 Links

Pseudomonas aeruginosa PlsX protein homolog (plsX) gene, partial cds; and malonyl-CoA:acyl carrier protein transacylase (fabD), 3-oxoacyl-acyl carrier protein reductase (fabG), acyl carrier protein (acpP), and 3-oxoacyl-acyl carrier protein synthase II (fabF) genes, complete cds
gi|2738151|gb|U91631.1|PAU91631[2738151]

☐ **3:** U39441 Links

Vibrio harveyi malonyl-CoA:ACP transacylase (fabD) gene, partial cds, and 3-ketoacyl-ACP reductase (fabG), acyl carrier protein (acpP), 3-ketoacyl-ACP synthase II (fabF) and aminodeoxychorismate lyase (pabC) genes, complete cds
gi|1173839|gb|U39441.1|VHU39441[1173839]

NCBI Nucleotide

Entrez PubMed Nucleotide Protein Genome Structure PMC Taxonomy Bc

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☐ **1:** U24241 Links

Sorangium cellulosum acyl-CoA dehydrogenase (sorE), beta-hydroxybutyryl-CoA dehydrogenase (sorD), putative methoxymalonyl-CoA synthase (sorC), reductase (sorR), soraphen polyketide synthase A (sorA), soraphen polyketide synthase B (sorB), O-methyltransferase (sorM), beta-mannanase, and xylanse-arabinofuranosidase bifunctional enzyme genes, complete cds; and unknown genes
gi|13346872|gb|U24241.2|SCU24241[13346872]

☐ **2:** U37088 Links

Simmondsia chinensis beta-ketoacyl-CoA synthase mRNA, complete cds
gi|1045613|gb|U37088.1|SCU37088[1045613]

☐ **3:** U75685 Links

Mycobacterium bovis acyl-CoA synthase gene, complete cds
gi|1658530|gb|U75685.1|MBU75685[1658530]

☐ **4:** S73733 Links

ACBP/DBI=acyl CoA-binding protein (ACBP)/diazepam-binding inhibitor (DBI)-endozepine homolog [Anas platyrhynchos=ducks, mallard, Genomic, 5191 nt]
gi|765222|bbm|351962|bbs|156494|gb|S73733.1|S73733[765222]

NCBI Nucleotide

Entrez PubMed Nucleotide Protein Genome Structure PMC Taxonomy Bc

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Items 1-10 of 18 Page 1 of 2 Next

1: U92878 Links
Garcinia mangostana acyl-ACP thioesterase (FatB1) mRNA, nuclear gene encoding chloroplast protein, complete cds
gi|1930080|gb|U92878.1|GMU92878[1930080]

2: U92877 Links
Garcinia mangostana acyl-ACP thioesterase (FatA2) mRNA, nuclear gene encoding chloroplast protein, complete cds
gi|1930078|gb|U92877.1|GMU92877[1930078]

3: U92876 Links
Garcinia mangsotana acyl-ACP thioesterase (FatA1) mRNA, nuclear gene encoding chloroplast protein, complete cds
gi|1930076|gb|U92876.1|GMU92876[1930076]

4: U56104 Links
Cuphea wrightii acyl-ACP thioesterase (FatB2) mRNA, complete cds
gi|1336007|gb|U56104.1|CWU56104[1336007]

5: U56103 Links
Cuphea wrightii acyl-ACP thioesterase (FatB1) mRNA, complete cds
gi|1336005|gb|U56103.1|CWU56103[1336005]

6: U38189 Links
Cuphea palustris thioesterase (FatB2) mRNA, complete cds
gi|1215719|gb|U38189.1|CPU38189[1215719]

7: U38188 Links
Cuphea palustris thioesterase (FatB1) mRNA, complete cds
gi|1215717|gb|U38188.1|CPU38188[1215717]

8: U31813 Links
Cinnamomum camphora acyl-ACP thioesterase mRNA, complete cds
gi|1143155|gb|U31813.1|U31813[1143155]

9: U17076 Links
Cuphea hookeriana 16:0-ACP thioesterase preprotein (Ch FatB1) mRNA,

complete cds
gi|758700|gb|U17076.1|CHU17076[758700]

10: U17098 Links
Brassica rapa acyl-ACP thioesterase Br FatA1 (FatA1) mRNA, complete cds
gi|595956|gb|U17098.1|BRU17098[595956]

Items 1-10 of 18 Page 1 of 2 Next

Display Summary Show: 10 Send to Text

Jul 27 2004 06:47:37

> *Bouquin, et al., "Resistance to trifluoroperazine, a calmodulin
> inhibitor, maps to the fabD locus in Escherichia coli," Mol Gen Genet. A1
> 246(5):628-37 (1995).

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ORIGINAL PAPER

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Resistance to trifluoroperazine, a calmodulin inhibitor, maps to the *fabD* locus in *Escherichia coli*

Received: 27 July 1994 / Accepted: 20 September 1994

Abstract A mutant, *tfpA1*, resistant to the calmodulin inhibitor trifluoroperazine (TFP) at 30°C, was isolated in *Escherichia coli*. The mutant showed a reduced growth rate at 30°C and was temperature sensitive (ts) at 42°C for growth, forming short filaments. The mutation was mapped to the 24 min region of the chromosome and the gene was cloned by complementation of the ts defect. Subsequent subcloning, complementation analysis, marker rescue mapping and sequencing, identified *tfpA* as *fabD*, encoding the 35 kDa, malonyl-coenzyme A transacylase (MCT) enzyme, required for the initial step in the elongation cycle for fatty acid biosynthesis. Resistance to TFP may result from altered permeability of the cell envelope, although the mutant remained sensitive to other calmodulin inhibitors and to other antibacterial agents. Alternatively, resistance may be more indirect, resulting from alterations in intracellular Ca^{++} levels which affect the activity of the TFP target in some way.

Key words *Escherichia coli* · Calmodulin
Trifluoroperazine · Cell division

Introduction

Calcium has been implicated in the regulation of a number of phenomena, ranging from neurotransmission to skeletal muscle contraction in eukaryotes (reviewed in Rasmussen and Rasmussen 1990). Some cell cycle events in eukaryotes are also controlled by calcium (Whitaker and Patel 1990; Lorca et al. 1993). In budding yeast, a growing body of evidence now suggests that calcium may play a key role in the regulation of some aspects of growth and the cell cycle (reviewed in Anraku et al. 1991). For example, Iida et al. (1990) have shown that calcium-starved *Saccharomyces cerevisiae* cells arrest primarily at the G2/M boundary, although entry into the DNA synthesis (S) phase was also delayed.

We have previously proposed (Norris et al. 1988; Holland et al. 1990) that, in bacteria, as in eukaryotes, calcium could play a predominant role in the control of growth and the cell cycle. In this model, a single calcium flux, occurring at a specific cell mass, would trigger all the cell cycle events: DNA replication, nucleoid segregation and cell division.

In prokaryotes, as in eukaryotes, calcium has been implicated in the regulation of many aspects of cell physiology. For example, the regulation of pathogenicity in *Yersinia* species or sporulation in *Bacillus subtilis* are controlled by calcium (see Holland et al. 1990; Norris et al. 1991). Calcium is also required for growth of *Escherichia coli* L-forms (Onoda et al. 1992) and for the growth of a number of bacteria including *Staphylococcus aureus*, *Clostridium perfringens*, *Streptococcus mutans* and *Streptococcus pneumoniae* (see Norris et al. 1991; Trombe et al. 1992). Chemotaxis in *E. coli* has been recently shown to be directly regulated by changes in the cytoplasmic calcium concentration (Tisa and Adler 1992). Furthermore, the calcium channel blocker ω-conotoxin inhibits *E. coli* chemotaxis (Tisa et al. 1993).

Both active calcium extrusion systems and calcium uptake systems have been described in bacteria (de Vrij et al. 1985; Rosen 1987; Preston et al. 1992; Van Veen et al. 1994). Calcium-binding proteins have been described in several prokaryotes (reviewed in Onek and Smith 1992; Ivey et al. 1993). In particular, the presence of three calcium binding proteins in *E. coli* has been reported by Harmon et al. (1985), and calmodulin-like activities have been detected in a number of bacteria (Onek and Smith 1992) and at least one of them (in *B. subtilis*) has been purified to near homogeneity (Fry et al. 1991) . In *E. coli*, a study has recently identified three calmodulin-like proteins whose synthesis is induced by EGTA treatment (Laoudj et al. 1994). However, the corresponding genes have been identified in only two cases, for protein S in *Myxococcus xanthus* (Inouye et al. 1983) and calery-

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Table 1 Strains and plasmids used in this study

Strains or plasmids	Genotype or construction	Source
D21	*proA 23, lac28, tsx81, his51, trp30, rpsL173, ampC1*	B. Bachmann
D22	*his51, trp30, proA23, lac28, strA173, ampA1, envA1, tsx81, tufA1*	Normark et al. (1969)
JM83	*ara, D(lac, proAB), rpsL, f80lacZDM15,(r_k^+, m_k^+)*	Laboratory collection
NFB216	As JM83, *pyrC, mdoA200*: :*Tn10*	Lacroix et al. (1989)
DS410T	*thi, ara, xyl, srl300*: :*Tn10, recA, minA minB, azi*	Laboratory collection
JC7623	*thr1, leuB6, D(gpt-proA)62, his64, argE3, thi1, ara14, lacY1, galK2, kdgK51, xyl5, mtl1, recB21, recC22, sbcB15, sbcC201, tsx33, rpsL31, supE44*	Laboratory collection
OMG3053	As D22, *tfpA1*	This study
OMG3080	As NFB216, *pyrC*$^+$, *tfpA1*	This study
OMG3081	As NFB216, *pyrC*$^-$, *mdoA*$^+$, *tfpA1*	This study
OMG3176	As JC7623, *mdoA200*: :*Tn10, tfpA1*	This study
pLG339	Kan^r, Tet^r	Stoker et al. (1982)
pBR322	Amp^r, Tet^r	Laboratory collection
Bluescript SK$^+$	Amp^r	Boehringer
pOMG3007	4.5 kb *Sau3A* genomic fragment, carrying the *tfpA*$^+$ gene, inserted into the *Bam*HI site of pLG339	This study
pOMG3010	Deletion of the 3.3 kb *Sal*I-*Sal*I fragment of pOMG3007	This study
pOMG3011	Deletion of the 1.8 kb *Sph*I-*Sph*I fragment of pOMG3007	This study
pOMG3014	Deletion of the 0.9 kb *Sph*I-*Pst*I fragment of pOMG3011	This study
pOMG3016	1.5 kb *Eco*RV-*Eco*RV fragment of pOMG3011 inserted into the *Eco*RV site of pBR322	This study
pOMG3018	1.5 kb *Sph*I-*Sal*I fragment of pOMG3011 inserted into the *Sph*I-*Sal*I sites of pLG339	This study
pOMG3020	3.5 kb *Eco*RI-*Nhe*I fragment of pOMG3011 inserted into the *Eco*RI-*Nhe*I sites of pLG339	This study
pOMG3022	1.2 kb *Eco*RI-*Sal*I fragment of pOMG3011 inserted into the *Eco*RI-*Sal*I sites of Bluescript SK$^-$	This study
pOMG3032	1.3 kb *Eco*RI-*BstE*II (filled with Klenow) fragment of pOMG3011 inserted into the *Eco*RI-*Eco*RV sites of pBR322	This study
pOMG3033	0.9 kb *Sal*I-*Stu*I fragment of pOMG3011 inserted into the *Sal*I-*Eco*RV sites of pBR322	This study

[a] r Resistance to *Kan* kanamycin, *Tet* tetracycline, *Amp* ampicillin

thrin in *Saccharopolyspora erythraeus* (Swan et al. 1987). Finally, the activity of *E. coli* proteins such as RecBC or EnvZ appears to be modulated by calcium (Rosamond et al. 1979; Rampersaud et al. 1991). Interestingly, the autophosphorylation activity of DnaK, a heat-shock protein involved in the *E. coli* cell cycle (Sakakibara 1988; Bukau and Walker 1989; Hwang et al. 1990; Mukherjee et al. 1993), is stimulated by calcium (Cegielska and Georgopoulos 1989). All these observations, therefore, point towards an important role for calcium in the regulation of *E. coli* growth and cell cycle events.

In order to investigate and perhaps define this role, *E. coli* mutants resistant to different "calcium agonists" and defective in cell cycle events, have been isolated and characterized in this laboratory (Casarégola et al. 1991). The *feeA1* and *feeB1* mutants, defective in cell division, are affected in the level or the activity of the rare $tRNA_3^{Leu}$ (Chen et al. 1991; Bouquin et al., submitted). These studies suggested that $tRNA_3^{Leu}$ may normally be limiting for expression of specific proteins involved in calcium homeostasis or indeed may play an additional role, coupling calcium homeostasis to the growth rate and or cell cycle events, through regulation of the level of $tRNA_3^{Leu}$ (Bouquin et al., submitted). The *verA1* mutation (Casarégola et al. 1991), conferring resistance to the calcium channel inhibitor verapamil and also defective in division, renders cells hypersensitive to the effects of EGTA, indicating defects in Ca^{++} transport. In addition, a mutant has been isolated from *B. subtilis*, which is resistant to an inhibitor of calcium-dependent protein kinase C (PKC), and has a reduced initiation mass for DNA synthesis, indicating an alteration in the timing of this cell cycle event (Séror et al. 1994).

In this study we have isolated a mutant resistant to trifluoroperazine (TFP), an inhibitor of calmodulin (Gietzen 1986), which has a reduced growth rate and aberrant cell division. Our results indicate that the *tfpA1* mutation is located in the fatty acid biosynthetic gene, *fabD*. We discuss the relationship of this mutation to the mechanism of fatty acid and membrane biogenesis and the basis of TFP resistance in *E. coli*.

Materials and methods

Bacterial strains and plasmids

The *E. coli* strains and the plasmids used in this study are listed in Table 1.

Conditions of growth

Liquid cultures were grown with vigorous shaking in Luria broth. M63 minimal salts supplemented with histidine, tryptophan and proline (M63AA). Cell mass was monitored by measuring absorbance at 600 nm (A^{600}) in a DMS90 spectrophotometer (Varian). For solid medium, culture media were supplemented with 1.5% agar.

Localisation of the *tfpA1* mutation

In order to map the *tfpA1* mutation, a P1 lysate was prepared from the *tfpA1* mutant (*tfpA1 pyrC*$^+$) and transduction was performed into the recipient NFB216 (*tfpA*$^+$ *pyrC*) as described in Miller (1972). The PyrC$^+$ transductants were selected on M63 plates and, amongst them, temperature resistant colonies were then screened by replica plating.

Isolation of TFP^r temperature-sensitive mutants

Several independent cultures of D22 were grown overnight in M63AA at 30°C. TFP dissolved in water was added to each culture to a final concentration of 150 μg/ml and 0.1 ml of each culture was plated onto an M63AA plate containing the same concentration of the drug. Some 750 mutants resistant to the drug, arising after incubation at 30°C, were purified and then screened by replica plating for temperature-sensitive mutants that failed to grow at 42°C on both LB and M63AA plates without drug.

Cloning of the *tfpA*$^+$gene

The cloning of the *tfpA*$^+$ gene was carried out essentially as described previously for the cloning of *feeB*$^+$ (Chen et al. 1991) and only the salient points will be mentioned here. An *E. coli* genomic bank was previously constructed in pLG339, a low-copy-number plasmid (Stoker et al. 1982). This bank was transformed into the *tfpA1* mutant and clones growing at 42°C were selected. The plasmid DNA from these clones was extracted and used to retransform the *tfpA1* mutant at 30°C. All these transformants then grew at 42°C without selection, confirming that the recombinant plasmid complemented the temperature sensitivity of the mutant.

Preparation and labelling of DNA probes for Southern blotting and dot-blotting

In order to localize the pOMG3007, *tfpA*$^+$ DNA on the *E. coli* chromosome, the plasmid was cut by *Eco*RI and the 3.7 kb band was purified from an 0.8% agarose gel using the Gene Clean kit (Ozyme, France). The DNA was labelled in vitro with [^{32}P]dCTP (Amersham International, specific activity 3000 Ci/mmol) using the Random Primer Labelling kit. The radiolabelled probe was then used to probe by Southern blotting (Sambrook et al. 1989) total *E. coli* DNA digested with *Bam*HI, *Hin*dIII, *Eco*RI, *Eco*RV, *Bgl*I, *Kpn*I, *Pst*I or *Pvu*II, or by dot-blotting (Sambrook et al. 1989) different total λ DNA from the ordered *E. coli* bank (Kohara et al. 1987).

Minicell analysis

Minicells were prepared by three successive centrifugations in sucrose gradients, as described in Stoker et al. (1984). After being resuspended in 50 ml of M63, cells were labelled for 30 min at 37°C with 10 mCi/ml of [^{35}S]methionine (Amersham International, specific activity 1000 Ci/mmol). The samples were analysed by SDS-PAGE in a 12% polyacrylamide gel and then autoradiographed.

Sequencing of the *tfpA* gene

In order to sequence the *tfpA* gene, the 1.2 kb *Eco*RI-*Sal*I restriction fragment from pOMG3011 was subcloned into Bluescript SK$^-$. The sequence of this insert was then determined from the universal primer by double-strand sequencing, performed with Sequenase version 2.0 (USB), according to the manufacturer's instructions.

In order to check that the pOMG3011 insert was identical to the *fabD*$^+$, *fabG*$^+$, *acpP*$^+$ region, a 17mer oligonucleotide (CGGCCCGGGCAAAGTGC) was used as a primer for double-strand sequencing of the pOMG3011 insert DNA. Double-strand sequencing was performed with Sequenase version 2.0 (USB), according to the manufacturer's instructions.

Marker rescue experiments

In order to localize precisely the *tfpA1* mutation, 1 mg of pOMG3032 (*fabD*$^+$), pOMG3033 (*fabG*$^+$) or pBR322 (vector) was linearized by digestion by *Nhe*I and transformed by electroporation (Sambrook et al. 1989) into the recipient *recBC sbcBC tfpA1* strain OMG3176. Cells were plated at 30°C on LB plates to determine the titre of the cultures, and at 42°C to determine the fraction of temperature resistant cells within each culture.

DNA manipulations.

All other DNA manipulations were performed according to Sambrook et al. (1989).

Results

Isolation of mutants resistant to trifluoroperazine

We have previously isolated mutants resistant to several calcium antagonists using the hyperpermeable strain N43 (Chen et al. 1991), which carries the *acrA1* mutation, responsible for increased sensitivity to a wide variety of drugs (Nakamura and Suganuma 1972; Ma et al. 1993). However, we considered that the type of mutations recovered might be influenced by the *acrA1* mutation. Since we wished to obtain the widest spectrum of mutations, an alternative hyperpermeable strain was chosen for this study. The *envA1* mutation carried by strain D22 causes hypersensitivity to both hydrophobic and hydrophilic agents (Normark et al. 1969).

Sensitivity of D22 to trifluoroperazine (TFP), a calmodulin inhibitor, was tested by adding different concentrations of TFP to an exponentially growing culture of D22 at 30°C in rich medium. At 20 μg/ml, D22 growth was seriously impaired. Total inhibition of growth was observed at 50 μg/ml (Fig. 1 top). In contrast, the *envA*$^+$ parental strain was resistant to TFP, even at a concentration as high as 100 μg/ml (data not shown).

For the isolation of mutants resistant to TFP, independent cultures of D22, grown overnight in M63 medium supplemented with the appropriate amino acids (M63AA, see Materials and methods), were plated on M63AA plates containing TFP at a concentration of

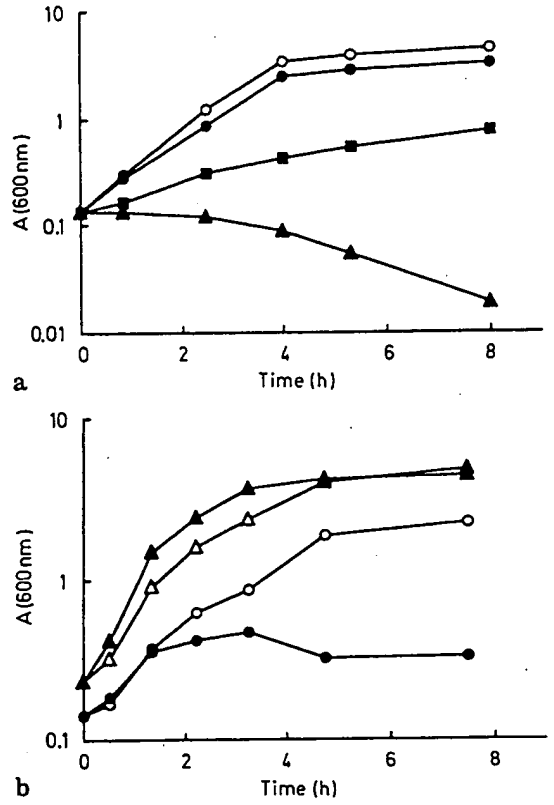

Fig. 1a,b Effect of trifluoroperazine (TFP) on D22 growth and growth of the *tfp1* mutant. a D22 was grown exponentially in LB medium at 30°C. At time zero, the culture was divided in four parts and TFP was added to a final concentration of 0 μg/ml (control, *open circles*), 10 μg/ml (*closed circles*), 20 μg/ml (*closed squares*) or 50 μg/ml. (*closed triangles*). Growth was followed by measuring the A_{600}. b D22 and the OMG3053, *tfpA1* mutant were grown exponentially in LB medium at 30°C. At time zero, a portion of the culture was transferred to 42°C whilst the other one was kept at 30°C. Growth was followed by measuring the A_{600}. D22, 30°C (*open triangles*), D22, 42°C (*closed triangles*), *tfpA1*, 30°C (*open circles*), *tfpA1*, 42°C (*closed circles*)

150 μg/ml and incubated at 30°C. Resistant mutants arose spontaneously at a frequency of approximately 10^{-8}.

Properties of the *tfpA1* mutant

Two mutants resistant to TFP, out of 750, were found to be also temperature sensitive. Importantly, none had regained increased resistance to SDS or ampicillin, indicating that they still carried the *envA1* mutation (Normark et al. 1969). In addition, they were still sensitive to other calmodulin inhibitors, such as 48/80 and W7.

The growth curve for one of these two mutants, designated *tfpA1*, is shown in Fig. 1 (bottom). The growth rate of the *tfpA1* mutant in LB medium is markedly reduced at 30°C, with a doubling time increased by approximately 50% (60 min vs 40 min). After *tfpA1* was shifted to 42°C, the mass continued to increase, although at a reduced rate, by 150% over 3 h. It is important to note that no general cell lysis occurred in the culture of the mutant kept at 42°C for several hours, suggesting that the integrity of the envelope was maintained in the mutant at the restrictive temperature.

The morphology of the mutant strain was also examined by phase contrast microscopy. The *tfpA1* cells were very heterogenous with respect to length and chain formation even at 30°C. However, since *envA1* cells, the parent of the *tfpA* mutant, often form chains (Normark et al. 1969), the potential effects of the *tfpA1* mutation upon cell division had to be examined in an $envA^+$ background. The *tfpA1* mutation was therefore moved by cotransduction with the $pyrC^+$ locus (see below) into strain NFB216 ($tfpA^+$ *pyrC*; Lacroix et al. 1989). We detected the presence of the mutation by screening for temperature sensitivity.

As shown in Fig. 2, in the $envA^+$ background, *tfpA* cells at the permissive temperature were very similar to the parental strain but the length of the majority of the cells increased at least twofold with incubation at the non-permissive temperature, 42°C, indicating a defect in division frequency as well as a severe effect on growth. However, the effect on division was apparently less dramatic than in the $envA^-$ background.

Temperature-resistant revertants (about 300) of the *tfpA1* mutant in the D22 background were isolated at a frequency of about 0.5×10^{-8}. All of the revertants were found to be also sensitive to TFP. This demonstrated that a single mutation resulted in both the temperature sensitivity and the resistance to the drug in the *tfpA1* mutant.

Cloning and localization of the $tfpA^+$ gene

A genomic bank of *E. coli* was previously constructed in pLG339, a low-copy-number plasmid (Chen et al. 1991). This bank was used to isolate recombinant plasmids that could complement the temperature sensitivity of the *tfpA1* mutant. Three such plasmids were isolated and were shown to be different from each other by Southern blotting (data not shown). However, two of these plasmid clones failed to restore drug sensitivity and presumably carried extragenic suppressors. The third clone, designated pOMG3007, complemented both phenotypes of the *tfpA1* mutant, and was therefore very likely to encode the $tfpA^+$ gene.

The 4.5 kb insert of pOMG3007 was localized by Southern blotting to the 1160–1170 kb region of the restriction map of the *E. coli* chromosome (Kohara et al. 1987), i.e. 24.5 min on the genetic map, as described in Materials and methods. This localization was confirmed by hybridization of the pOMG3007 insert with the corresponding phages, λ 234 and 235, from the ordered miniset bank of Kohara and co-workers (Kohara et al. 1987), in dot-blot experiments (see Materials and methods). In addition, the *tfpA1* mutation could be cotransduced (6%) by phage P1, together with the $pyrC^+$ locus (by screening for temperature sensitivity), into the recipient NFB216 ($pyrC$ $tfpA^+$) strain. In agreement with the physical mapping, this suggested that the *tfpA1* mutation

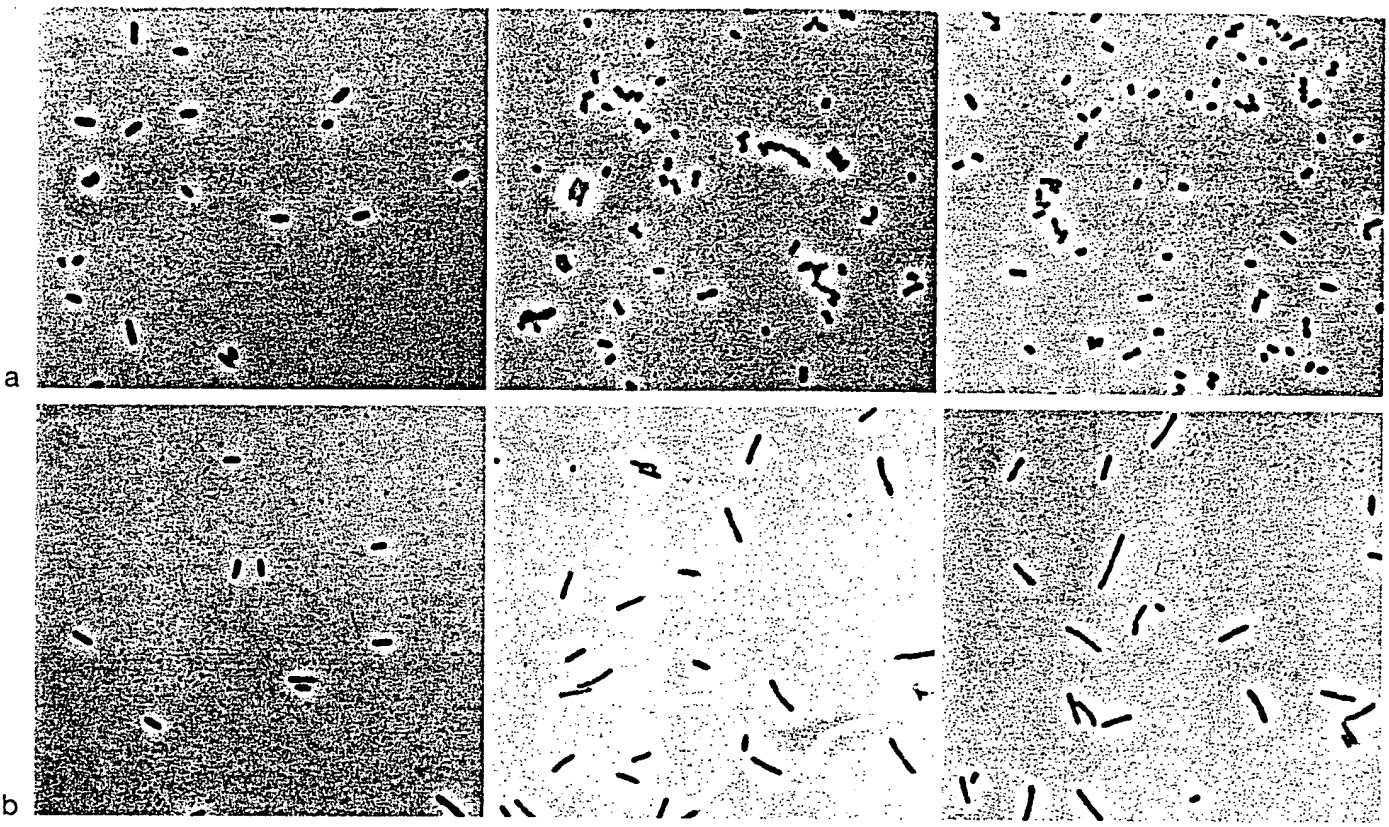

Fig. 2a,b Phase contrast microscopy of D22 and *tfpA1* after shift to 42°C. The parental strain D22 (a) and OMG3053 (*tfpA1*) (b) growing exponentially in Luria broth at 30°C were shifted to 42°C at time zero (left panels) and sampled after 1 h (middle panels) and 2 h (right panels), fixed and photographed under phase contrast

mapped either to the 22 min region or the 24 min region of the chromosome. In order to localize more precisely the mutation, a three-point cross was performed. A P1 lysate was prepared from the *tfpA1* mutant (*tfpA1 mdoA*$^+$ *pyrC*$^+$) and transduction was performed (see Materials and methods) with this lysate into the recipient NFB216 (*tfpA*$^+$ *mdoA*::Tn*10 purC*). The *tfpA1* mutation was 6% cotransducible with *pyr*$^+$ and 4% with *mdoA*$^+$. Since *mdoA* is located at 23 min and *pyrC* at 23.5 min on the *E.coli* genetic map, we could unequivocally assign the *tfpA1* mutation to the 24–24.5 min region, thus demonstrating that the pOMG3007 insert contains the *tfpA*$^+$ gene and not a suppressor.

Mapping of the *tfpA* gene within the pOMG3007 insert

In order to localize precisely the *tfpA* gene within the pOMG3007 insert, a deletion/subcloning analysis was carried out. Various deletions or subclones were generated from pOMG3007 (see Materials and methods), utilizing known restriction sites, and these were tested for their ability to restore temperature resistance and drug sensitivity in the *tfpA1* mutant (in the *envA1* background). As shown in Fig. 3, this enabled us to map one end of the *tfpA* gene downstream of the central *Eco*RI site, with the other end extending beyond the *Xho*I site; compare pOMG3020, which complements both mutant phenotypes, with pOMG3010 and pOMG3014 which do not complement. In order to determine the right hand border of *tfpA* more precisely, attempts were made to construct subclones with the right endpoint at the *Eco*RV site or at the *Kpn*I site. However, such constructs were impossible to recover, suggesting that they may be toxic.

Sequencing of the region of the *tfpA* gene

The *Eco*RI-*Sal*I restriction fragment of pOMG3011, which complements the mutant phenotypes (see Fig. 3), was cloned into Bluescript SK$^+$ and sequenced by double-strand sequencing, as described in Materials and methods. The resulting data, when compared with sequences in the EMBL database, were identical to the recently sequenced *fabH* and *fabD* genes (Tsay et al. 1992; Verwoert et al. 1992). As shown in Fig. 3, sequence data indicated that the *Sal*I-*Eco*RV fragment of pOMG3011 overlaps the end of the *fabD* gene and the beginning of the downstream *fabG* gene (Rawlings and Cronan 1992).

The *fabH*, *fabD* and *fabG* genes are part of a fatty acid biosynthesis gene cluster which comprises, clockwise on the chromosome, at least the following genes: *plsX - fabH - fabD - fabG - acpP*$^+$ *- fabF* (Oh and Larson,

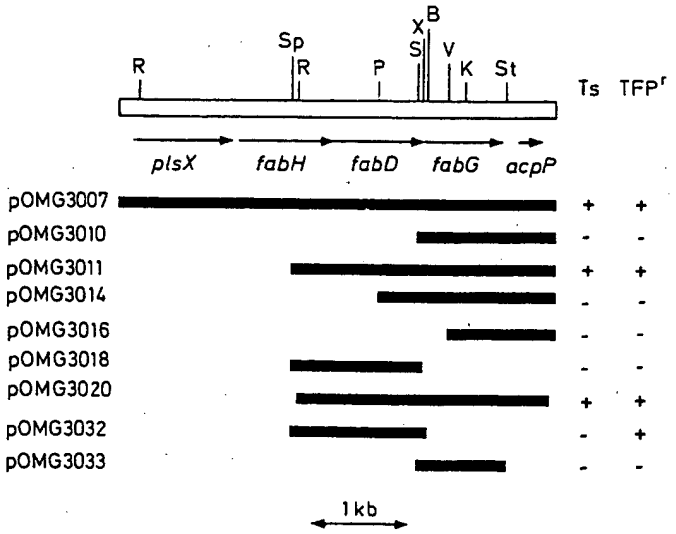

Fig. 3 Localization of the *tfpA* gene by complementation, deletion analysis of pOMG3007. Deletions and subclones of the *tfpA* region were constructed and analysed at 42°C for complementation of the temperature sensitivity and at 30°C for drug sensitivity. In the upper part of the figure, the region of the chromosome containing the *tfpA* gene is indicated as an *open box* (the restriction sites are: R, *Eco*RI; Sp, *Sph*I; P, *Pst*I; Sm, *Sma*I; S, *Sal*I; X, *Xho*I; B, *Bst*EII; V, *Eco*RV; K, *Kpn*I; St, *Stu*I) The positions of the different genes mapping in this region are indicated below. In the lower part of the figure, are shown the inserts of pOMG3007 and several of its derivatives. Ts^+ complementing the temperature sensitivity of the mutant (growth at 42°C), Ts^- not complementing the temperature sensitivity of the mutant (no growth at 42°C), TFP^{r+} complementing the resistance of the mutant (sensitive to TFP at 30°C), TFP^{r-} not complementing the resistance of the mutant (still resistant to TFP at 30°C)

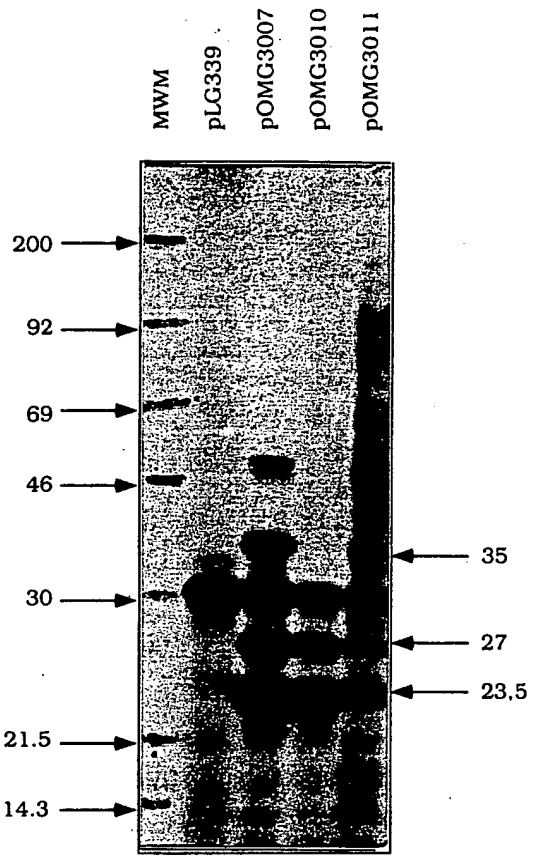

Fig. 4 Visualization of the *tfpA* product. The DS410T strain (Stoker et al. 1984) was transformed with either pLG339, pOMG3007, pOMG3010 or pOMG3011. Minicells were prepared and proteins labelled with [^{35}S]methionine. The samples were then analysed in a 12% polyacrylamide gel and autoradiographed. The sizes of the molecular weight markers are indicated on the left and the sizes of the three proteins encoded by pOMG3007 and its derivatives are shown on the right

EMBL database; Tsay et al. 1992; Verwoert et al. 1992; Rawlings and Cronan 1992). The insert of pOMG3007 therefore contains *plsX*, *fabH*, *fabD*, *fabG* and $acpP^+$ and, possibly, the beginning of *fabF*, as inferred from a comparison of the restriction maps (Fig. 3).

The FabH, FabD, FabG and ACP proteins have been expressed previously from the cloned genes and shown to migrate as 35 kDa, 32 kDa, 26.5 kDa and 20 kDa proteins, respectively (Tsay et al. 1992; Verwoert et al. 1992; Rawlings and Cronan 1992). In each case, the observed molecular weight was in good agreement with the corresponding gene product, except for ACP, an 8.8 kDa protein which migrates aberrantly.

Visualization of the *tfpA* gene product

The results of our deletion/subcloning analysis were all consistent with the hypothesis that *tfpA* was identical to *fabD*. Essentially, all the constructions which carried an interrupted *fabD* gene lost the capacity to complement the *tfpA1* temperature sensitivity and TFP resistance (Fig. 3). In order to confirm that *tfpA* and *fabD* were the same gene, we wished to visualize the *tfpA* gene product directly.

pOMG3007 and various derivatives of this clone were transformed into the minicell-producing strain DS410T (Stoker et al. 1984) and minicells were purified and labelled with [^{35}S]methionine as described in Materials and methods. The insert of the pOMG3007 plasmid appeared to encode at least three proteins of 35 kDa, 27 kDa and 23.5 kDa, respectively (Fig. 4). A doublet of proteins in the 50 kDa range could also be detected, although this was much less well expressed.

Since FabG and ACP migrate as 27 kDa and 20 kDa proteins, respectively (Rawlings and Cronan 1992), it was very likely that the pOMG3007-encoded 27 kDa and 23.5 kDa proteins were identical to the *fabG* and *acpP* gene products, respectively. Moreover, pOMG3010, which carries only the *fabG* and *acpP* genes, encoded only two proteins of 27 kDa and 23.5 kDa (Figs. 3, 4). Finally when the right-hand part of the pOMG3011 insert up to the *Eco*RV site, i.e. encompassing the *acpP* gene, was subcloned into the *Eco*RV site of pBR322, generating pOMG3016 (Fig. 3), a single protein of 23.5 kDa was expressed in minicells, which was heat-stable (data not shown), a property of ACP (Cronan and

Rock 1987). All our evidence were thus consistent with the 27 kDa and 23.5 kDa proteins corresponding to the *fabG* and *acpP* gene products.

The identity of the 35 kDa protein was initially less clear, since *fabH* and *fabD* encode 35 and 32 kDa proteins, respectively (Tsay et al. 1992; Verwoert et al. 1992). However, a deletion of the insert of the pOMG3007 plasmid up to the *Sph*I site (pOMG3011), which destroyed the *fabH* gene but left the *fabD* gene intact, still encoded the 35 kDa protein (Figs. 3, 4). Moreover, a deletion of the pOMG3007 insert up to the *Sal*I site, generating a subclone carrying only the *fabG* and *acpP* gene, namely pOMG3010 (Fig. 3), did not express the 35 kDa protein (Fig. 4). It therefore seems very likely that the 35 kDa protein is the *fabD* gene product. Similarly, since pOMG3011 (uniquely encoding the 35 kDa protein), unlike pOMG3010, was able to reverse both the temperature sensitivity and resistance to TFP, the results strongly suggest that the *tfpA* gene product is the 35 kDa protein, and that *tfpA* and *fabD* are identical.

The *tfpA1* mutation affects the *fabD* gene

Although all our data were consistent with the *tfpA1* mutation affecting the *fabD* gene, we were not able to construct a clone with an intact *fabD* and an interrupted *fabG* gene, perhaps because the product of the latter is toxic (see above). In order to obtain definitive evidence that *tfpA* is identical to *fabD*, the 1.3 kb *Eco*RI-*Bst*EII restriction fragment from the pOMG3011 insert, corresponding to the *fabD* gene, was subcloned into pBR322 (see Materials and methods), generating plasmid pOMG3032. In addition, the 0.9 kb *Sal*I-*Stu*I fragment of the pOMG3011 insert, which bears only the *fabG* gene, was inserted into the *Sal*I and *Eco*RV sites of pBR322, generating plasmid pOMG3033 (see Materials and methods). Both pOMG3032 and pOMG3033, and pBR322 as a control, were transformed into the *tfpA1* mutant. As expected, neither pBR322 nor pOMG3033 (*fabG*) were able to restore temperature resistance, nor to confer drug sensitivity on the *tfpA1* mutant (Fig. 3). As expected pOMG3032 (*fabD*) reversed the resistance to TFP, however, unexpectedly, it failed to restore to *tfpA1* the capacity to grow at 42°C.

In order to demonstrate unequivocally, therefore, that *tfpA1* affects the *fabD* gene, a different approach was required, that is, to map genetically the *tfpA1* mutation more precisely. The *tfpA1* mutation was moved by P1 transduction from OMG3080 (*mdoA*::Tn*10 tfpA*) into a *recBC sbcBC* strain, with initial selection for tetracycline-resistant colonies, followed by subsequent screening for temperature-sensitive clones. The resulting strain was designated OMG3176. Marker rescue experiments were then performed with either pOMG3032 (*fabD*), pOMG3033 (*fabG*) or pBR322 (vector): 1 μg of each plasmid was linearized by *Nhe*I, transformed into the OMG3176 (*tfpA1 recBC sbcBC*) strain and the fraction of temperature-resistant cells was determined (see Materials and methods). Both pOMG3033 (*fabG*) and pBR322 gave similar low frequencies of temperature-resistant colonies, i.e. approximately 10^{-8}, close to the reversion rate of approximately 0.5×10^{-8} (see above). In contrast, transformation with linearized pOMG3032 (*fabD*) yielded 32 temperature-resistant colonies, corresponding to a frequency of approximately 10^{-7}. This result strongly supported the idea that the *tfpA1* mutation, with its consequent effect on growth at high temperature, was located in the *fabD* gene. These results, combined with the results of complementation studies with pOMG3032 (*fabD*), indicate that temperature sensitivity was dominant under these conditions. It is important to note that this analysis was carried out in the OMG3176, (*envA*$^+$) strain, in which it was not possible to test the drug response of the recombinants since TFP resistance is not expressed in this background.

Discussion

In order to study the role of calcium in *E. coli* growth and the cell cycle, we had previously isolated mutants resistant to different calcium-binding protein inhibitors, including 48/80, W7 and verapamil (Casarégola et al. 1991; Chen et al. 1991; Bouquin et al., submitted; S. Bernard and A. Jacq, personal communication). In this study, we have isolated an *E. coli* mutant resistant to TFP, an inhibitor of calmodulin (Gietzen 1986). This mutant, designated *tfpA1*, was also temperature sensitive, being defective in division at 42°C, indicating that it was affected in an essential function. This conclusion was reinforced by the finding that, even at 30°C, *tfpA1* grew much more slowly than its parent.

Genetic analysis of the *tfpA1* mutation mapped the gene close to 24 min. A clone, pOMG3007, was isolated by complementation of the temperature sensitivity of *tfpA1*, which also complemented the drug resistance and the morphological defects (data not shown) of the mutant. The insert of pOMG3007 was shown to hybridize with λ 234 and 235 from the ordered bank of *E. coli* (Kohara et al. 1987) corresponding to the 24 min region of the chromosome. This result demonstrated that pOMG3007 carried the *tfpA*$^+$ gene and not a suppressor.

Sequencing of the pOMG3007 insert revealed the presence of five genes, recently deposited in the EMBL database, which are in the following order on the chromosome: *plsX*$^+$, *fabH*$^+$, *fabD*$^+$, *fabG*$^+$, *acpP*$^+$ (Oh and Larson, EMBL database; Tsay et al. 1992; Verwoert et al. 1992; Rawlings and Cronan 1992). Only three proteins, of 35 kDa, 27 kDa and 23.5 kDa, encoded by the pOMG3007 insert, could be visualized in minicell experiments. None of these proteins appeared to be encoded by the *plsX*$^+$ or the *fabH*$^+$ genes, since all three polypeptides could still be detected with pOMG3011, a subclone of pOMG3007 which does not contain the *plsX*$^+$ and *fabH*$^+$ genes. The 23.5 kDa polypeptide was encoded by *acpP*$^+$, since it was the sole protein detected

with pOMG3016, which carries only this gene. On the other hand, since the 35 kDa protein was not observed whenever the *fabD*$^+$ gene was interrupted, it is very likely that this protein corresponds to the product of this gene. Finally, because the pOMG3010 insert (*fabD*$^+$ *acpP*$^+$) encodes the 27 kDa and 23.5 kDa proteins, the latter corresponding to the *acpP*$^+$ gene product, we tentatively assign the 27 kDa protein to the *fabG*$^+$ gene.

The results of deletion/subcloning analysis indicate that complementation of the phenotypes of *tfpA1* was blocked in the absence of an intact *fabD*$^+$ gene. Surprisingly, however, *fabD*$^+$ alone (pOMG3032), although complementing drug resistance, did not complement the temperature sensitivity of *tfpA1*. This raised the possibility that the mutant carried two mutations, although this seems unlikely since reversion analysis indicated a single mutation. Importantly, however, the marker rescue experiments with pOMG3032 (*fabD*$^+$), showed that recombination with the wild-type *fabD* alone (the *Eco*RI-*Bst*EII fragment) was sufficient to correct the temperature sensitive phenotype of the *tfpA1* mutant. We thus conclude, although it has not been unequivocally demonstrated, that the *tfpA1* mutant carries a single mutation in *fabD*, which under some conditions is dominant at 42°C.

The *fabD*$^+$ gene encodes the malonyl-coenzymeA-acyl carrier protein transacylase (MCT). This enzyme catalyzes the formation of malonyl-ACP from malonyl-coenzyme A and acyl carrier protein (ACP). A two-carbon unit from malonyl-ACP is subsequently added to a preexisting carbon chain esterified to ACP by a 3-ketoacyl-ACP-protein synthetase, the product of the *fabH*$^+$ gene (Tsay et al. 1992), in the first step of the elongation cycle of fatty acid biosynthesis (Cronan and Rock 1987).

A mutation affecting the *fabD*$^+$ gene was previously isolated (Harder et al. 1974). This mutation, *fabD89*, was an amber mutation, generating an inactive truncated enzyme. However, because of the presence in the mutant strain of a suppressor tRNA, a full-length protein could be synthesized, although the activity of the resulting, modified enzyme was temperature sensitive. As a result, the mutant strain failed to grow at high temperatures (Verwoert et al. 1992).

Although several lines of evidence indicated that the *tfpA1* mutation was located in the *fabD* gene, the situation may, however, be more complex since the temperature sensitivity of the mutant could not be complemented by the *fabD*$^+$ gene alone. Restoration of growth at 42°C required a plasmid carrying the three genes, *fabD*$^+$, *fabG*$^+$ and *acpP*$^+$. The normal stoichiometry of the three gene products might therefore be required for full expression and/or activity of FabD in a coordinated sequence of reactions, leading to full-length fatty acid chains. A more complex explanation for the inability of the *fabD* gene clone to complement temperature sensitivity could be that, for example, the *tfpA1* mutation causes reduced expression of *fabG* and *acpP* at 42°C. This is not excluded but seems unlikely to involve transcriptional control since there was no detectable reduction at 42°C of *fabG* or *acpP* mRNA, as detected by Northern blotting (unpublished results). Interestingly, however, overexpression of the DNA-binding protein FadR, which is a transcriptional activator of at least one fatty acid biosynthesis gene, *fabA*$^+$, encoding 3-hydroxydecanoyl-ACP deshydrase (Henry and Cronan 1991; 1992; DiRusso et al. 1993), suppresses temperature sensitivity in *tfpA* mutants (A. R. Stuitje, personal communication). This result suggests that enhanced levels of the mutant enzyme are sufficient to overcome any deficit in long chain fatty acid synthesis.

As indicated above, the *tfpA1* mutation, like the *fabD89* mutation (Harder et al. 1974), might be expected to result in an altered lipid composition in the membrane, in particular a reduction in long chain fatty acids. Drug resistance could arise, therefore, as a result of an altered permeability of the cell envelope. This effect would, nevertheless, have to be relatively specific since the cells remained unchanged in sensitivity to, for example, the calmodulin inhibitors, W7 and 48/80. Another possibility is that *tfpA1*, like the *htrB* mutant (Karow et al. 1992) may be defective in the coupling between the growth rate and the rate of phospholipid synthesis. Directly or indirectly the mutation may lead to altered levels of intracellular Ca^{++} and disturbed regulation of cell division. If this were the case, then the *tfpA* gene would be a new member of a growing class of genes conferring sensitivity to "calcium metabolism" antagonists and involved in coupling growth to cell cycle events in both *E. coli* (Chen et al. 1991; Bouquin et al., submitted) and in *B. subtilis* (Séror et al. 1994).

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References

Anraku Y, Ohya Y, Iida H (1991) Cell cycle control by calcium and calmodulin in *Saccharomyces cerevisiae*. Biochim Biophys Acta 1093: 169–177

Bukau B, Walker GC (1989) Cellular defects caused by deletion of the *Escherichia coli dnaK* gene indicates roles for heat shock protein in normal metabolism. J Bacteriol 171: 2337–2346

Casarégola S, Chen M, Bouquin N, Norris V, Jacq A, Goldberg M, Margarson S, Tempête M, McKenna S, Sweetman H, Bernard S, McGurk G, Séror SJ, Holland IB (1991) Analysis of a myosin-like protein and the role of calcium in the *E. coli* cell-cycle. Res Microbiol 142: 201–207

Cegielska A, Georgopoulos C (1989) Functional domains of the *Escherichia coli* DnaK heat shock protein as revealed by mutational anlysis. J Biol Chem 264: 21122–21130

Chen MX, Bouquin N, Norris V, Casarégola S, Séror SJ, Holland IB (1991) A single base change in the acceptor stem of

*tr*NA$_3^{Leu}$ confers resistance upon *Escherichia coli* to the calmodulin inhibitor, 48/80. EMBO J 10:3113–3122

Cronan JE Jr, Rock CO (1987) Biosynthesis of membrane lipids. In: Neidhardt FC, Ingraham JB, Low KB, Magasanik B, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, DC, pp 474–497

De Vrij W, Bulthuis R, Postma E, Konings W (1985) Calcium transport in membrane vesicles of *B. subtilis*. J Bacteriol 164:1294–1300

DiRusso CC, Metzger AK, Heimert TL (1993) Regulation of transcription of genes required for fatty acid transport and unsaturated fatty acid biosynthesis in *Escherichia coli* by FadR. Mol Microbiol 7:311–322

Fry IJ, Becker-Hapak M, Hageman JH (1991) Purification and properties of an intracellular calmodulin-like protein from *Bacillus subtilis* cells. J Bacteriol 173:2506–2513

Gietzen K (1986) Pharmacological regulation of the activity of calmodulin. In: Bader H, Gietzen K, Rosenthal J, Rüdel R, Wolf HU (eds) Intracellular calcium regulation. Manchester University Press, Manchester, pp 405–421

Harmon AC, Prasher D, Cormier MJ (1985) High affinity calcium binding proteins in *E. coli*. Biochem Biophys Res Commun 127:31–36

Harder ME, Ladenson RC, Schimmel SD, Silbert DF (1974) Mutants of *Escherichia coli* with temperature-sensitive malonyl coenzyme A-acyl carrier protein transacylase. J Biol Chem 249:7468–7475

Henry MF, Cronan JE Jr (1991) *Escherichia coli* transcription factor that both activates fatty acid synthesis and represses fatty acid degradation. J Mol Biol 222:843–849

Henry MF, Cronan JE Jr (1992) A new mechanism of transcriptional regulation: release of an activator triggered by small molecule binding. Cell 70:671–679

Holland IB, Casarégola S, Norris V (1990) Cytoskeletal elements and calcium: do they play a role in the *E. coli* cell cycle? Res Microbiol 141:131–136

Hwang DS, Crooke E, Kornberg A (1990) Aggregated *dnaA* protein is dissociated and activated for DNA replication by phospholipase or *dnaK* protein. J Biol Chem 265:19244–19248

Iida H, Sakaguchi, Yagawa Y, Anraku Y (1990) Cell cycle control by Ca^{2+} in *Saccharomyces cerevisiae*. J Biol Chem 265:21216–21222

Inouye S, Franceschini T, Inouye M (1983) Structural similarities between the development-specific protein S from a gram-negative bacterium, *Myxococcus xanthus*, and calmodulin. Proc Natl Acad Sci USA 80:6829–6833

Ivey DM, Guffanti AA, Zemsky J, Pinner E, Karpel R, Padan E, Schuldiner S, Krulwich TA (1993) Cloning and characterization of a putative Ca^{2+}/H^+ antiporter gene from *Escherichia coli* upon functional complementation of Na^+/H^+ antiporter-deficient strains by the overexpressed gene. J Biol Chem 268:11296–11303

Karow M, Fayet O, Georgopoulos C (1992) The lethal phenotype caused by null mutations in the *Escherichia coli htrB* gene is suppressed by mutations in the accBC operon, encoding two subunits of acetyl coenzyme A carboxylase. J Bacteriol 174:7407–7418

Kohara Y, Akiyama K, Irono K (1987) The physical map of the whole *Escherichia coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell 50:495–508

Lacroix J-M, Tempête M, Menichi B, Bohin J-P (1989) Molecular cloning and expression of a locus (*mdoA*) implicated in the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. Mol Microbiol 3:1173–1182

Laoudj D, Andersen CL, Bras A, Goldberg M, Jacq A, Holland IB (1994) EGTA induces the synthesis in *Escherichia coli* of three proteins that cross-react with calmodulin antibodies. Mol Microbiol 13:445–457

Lorca T, Cruzalegui FH, Fesquet D, Cavadore J-C, Méry J, Means A, Dorée M (1993) Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. Nature 366:270–273

Ma D, Cook DN, Alberti M, Pon NG, Nikaido H, Hearst JE (1993) Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J Bacteriol 175:6299–6313

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Mukherjee A, Dai K, Lutkenhaus J (1993) *Escherichia coli* cell division FtsZ is a guanine nucleotide binding protein. Proc Natl Acad Sci USA 90:1053–1057

Nakamura H, Suganuma A (1972) Membrane mutation associated with sensitivity to acriflavine in *Escherichia coli*. J Bacteriol 110:329–335

Normark S, Bowman H, Matsson E (1969) Mutant of *E. coli* with anomalous cell division and ability to decrease episomally and chromosomally mediated resistance to ampicillin and several other antibiotics. J Bacteriol 97:1334–1342

Norris V, Séror SJ, Casarégola S, Holland IB (1988) A single calcium flux triggers chromosome replication, segregation and septation in bacteria: a model. J Theor Biol 134:341–350

Norris V, Chen M, Goldberg M, Voskuil J, McGurk G, Holland IB (1991) Calcium in bacteria: a solution to which problem? Mol Microbiol 5:775–778

Onek LA, Smith RJ (1992) Calmodulin and calcium mediated regulation in prokaryotes. J Gen Microbiol 138:1039–1049

Onoda T, Oshima A, Fukunaga N, Nakatani A (1992) Effect of Ca^{2+} and K^+ on the intracellular pH of an *Escherichia coli* L-form. J Gen Microbiol 138:1265–1270

Preston RR, Saimi Y, Martinac B, Kung C (1992) Genetic analysis of ion channels of prokaryotes and lower eukaryotes. Curr Biol 2:780–784

Rampersaud A, Utsumi R, Delgado J, Forst SA, Inouye M (1991) Ca^{2+}-enhanced phosphorylation of a chimeric protein kinase involved with bacterial signal transduction. J Biol Chem 266:7633–7637

Rasmussen H, Rasmussen JE (1990) Calcium as intracellular messenger: from simplicity to complexity. Curr Top Cell Regul 31:1–109

Rawlings M, Cronan JE Jr (1992) The gene encoding *Escherichia coli* acyl carrier protein lies within a cluster of fatty acid biosynthetic genes. J Biol Chem 267:5751–5754

Rosamond J, Telander KM, Linn S (1979) Modulation of the action of the *recBC* enzyme of *Escherichia coli* K-12 by Ca^{2+}. J Biol Chem 254:8646–8652

Rosen BP (1987) Bacterial calcium transport. Biochim Biophys Acta 906:101–110

Sakakibara Y (1988) The *dnaK* gene of *Escherichia coli* functions in initiation of chromosome replication. J Bacteriol 170:972–979

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Séror SJ, Casarégola S, Vannier F, Zouari N, Dahl M, Boye E (1994) A mutant cysteinyl-tRNA synthetase affecting timing of chromosomal replication initiation in *B. subtilis* and conferring resistance to a protein kinase C inhibitor. EMBO J 13:2472–2480

Stoker NG, Fairweather NF, Spratt BG (1982) Versatile low copy number plasmid vector for cloning in Escherichia coli. Gene 18:335–341

Stoker NG, Pratt JM, Holland IB (1984) In vivo gene expression systems in prokaryotes. In: Hames BD, Higgins SJ (eds) Transcription and translation, a practical approach. IRL Press, Oxford, UK, pp 153–177

Swan DG, Hale RS, Dhillon N, Leadlay PF (1987) A bacterial calcium-binding protein homologous to calmodulin. Nature 329:84–85

Tisa LS, Adler J (1992) Calcium ions are involved in *Escherichia coli* chemotaxis. Proc Natl Acad Sci USA 89:11804–11808

Tisa LS, Olivera BM, Adler J (1993) Inhibition of *Escherichia coli* chemotaxis by ϖ-conotoxin, a calcium ion channel blocker. J Bacteriol 175: 1235–1238

Trombe M-C, Clavé C, Manias J-M (1992) Calcium regulation of growth and differentiation in *Streptococcus pneumoniae*. J Gen Microbiol 138: 77–84

Tsay J-T, Oh W, Larson TJ, Jackowski S, Rock CO (1992) Isolation and characterization of the b-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. J Biol Chem 267: 6807–6814

Van Veen HW, Abee T, Korststee GJJ, Konings WN, Zehnder AJB (1994) Translocation of metal phosphate via the phosphate inorganic transport system of *Escherichia coli*. Biochemistry 33: 1766–1770

Verwoert IGS, Verbree EC, Van Der Linden KH, Nijkamp HJJ, Stuitje AR (1992) Cloning, nucleotide sequence, and expression of the *Escherichia coli fabD* gene, encoding malonyl-coenzyme A-acyl carrier protein transacylase. J Bacteriol 174: 2851–2857

Whitaker M, Patel R (1990) Calcium and cell cycle control. Development 108: 525–542

>
> *Boynton, et al., "Cloning, sequencing, and expression of clustered genes
> encoding beta-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase,
> and butyryl-CoA dehydrogenase from Clostridium acetobutylicum ATCC 824," J
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Cloning, Sequencing, and Expression of Clustered Genes Encoding β-Hydroxybutyryl-Coenzyme A (CoA) Dehydrogenase, Crotonase, and Butyryl-CoA Dehydrogenase from *Clostridium acetobutylicum* ATCC 824

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The enzymes β-hydroxybutyryl-coenzyme A (CoA) dehydrogenase (BHBD), crotonase, and butyryl-CoA dehydrogenase (BCD) from *Clostridium acetobutylicum* are responsible for the formation of butyryl-CoA from acetoacetyl-CoA. These enzymes are essential to both acid formation and solvent formation by clostridia. Clustered genes encoding BHBD, crotonase, BCD, and putative electron transfer flavoprotein α and β subunits have been cloned and sequenced. The nucleotide sequence of the *crt* gene indicates that it encodes crotonase, a protein with 261 amino acid residues and a calculated molecular mass of 28.2 kDa; the *hbd* gene encodes BHBD, with 282 residues and a molecular mass of 30.5 kDa. Three open reading frames (*bcd*, *etfB*, and *etfA*) are located between *crt* and *hbd*. The nucleotide sequence of *bcd* indicates that it encodes BCD, which consists of 379 amino acid residues and has high levels of homology with various acyl-CoA dehydrogenases. Open reading frames *etfB* and *etfA*, located downstream of *bcd*, encode 27.2- and 36.1-kDa proteins, respectively, and show homology with the *fixAB* genes and the α and β subunits of the electron transfer flavoprotein. These findings suggest that BCD in clostridia might interact with the electron transfer flavoprotein in its redox function. Primer extension analysis identified a promoter consensus sequence upstream of the *crt* gene, suggesting that the clustered genes are transcribed as a transcriptional unit and form a *BCS* (butyryl-CoA synthesis) operon. A DNA fragment containing the entire *BCS* operon was subcloned into an *Escherichia coli-C. acetobutylicum* shuttle vector. Enzyme activity assays showed that crotonase and BHBD were highly overproduced in cell extracts from *E. coli* harboring the subclone. In *C. acetobutylicum* harboring the subclone, the activities of the enzymes crotonase, BHBD, and BCD were elevated.

The anaerobic, spore-forming bacterium *Clostridium acetobutylicum* has for some time been known for the ability to produce, through fermentation, the commercial solvents acetone and butanol (30). Recently, however, the more economical use of petrochemicals to produce these solvents has virtually eliminated the industrial fermentation process. However, such a process has remained of interest to researchers as a valid model for understanding the regulation and genetics of complex primary metabolism. With recent advances in genetic engineering, interest has focused again on the possibility that solvent-producing clostridial metabolism can be commercially viable (45).

β-Hydroxybutyryl-coenzyme A (CoA) dehydrogenase (BHBD), crotonase, and butyryl-CoA dehydrogenase (BCD) from *C. acetobutylicum* are enzymes in the central fermentation pathway which play a central role in both acid production and solvent production (30). A similar enzyme arrangement of 3-hydroxyacyl-CoA dehydrogenase (HAD), enoyl-CoA hydratase (ECH), and acyl-CoA dehydrogenase (ACD) is involved in the β oxidation of fatty acids in eukaryotes (Fig. 1A of reference 70), suggesting possible structural and mechanistically conserved elements. In addition, a multifunctional enzyme complex, comprising thiolase, HAD, ECH, epimerase, and isomerase, is encoded by fatty acid degradative operon *fadBA*, found in *Escherichia coli* (52, 57, 67, 68).

BHBD in clostridia catalyzes the reduction of acetoacetyl-CoA by NAD(P)H. This subprocess is the initial and necessary step toward the ultimate production of butyrate and butanol. The *hbd* gene, encoding BHBD from *C. acetobutylicum* P262 and *Clostridium difficile*, has already been cloned in *E. coli* and sequenced (47, 70). For *C. acetobutylicum* P262, *hbd* encodes a protein of 282 amino acids with a calculated molecular mass of 31 kDa, and for *C. difficile*, it encodes a protein of 281 residues with a molecular mass of 31 kDa.

Crotonase (EC 4.2.1.17), or ECH, from *C. acetobutylicum* NRRL-B-527 has been previously purified and characterized (60). Unlike the crotonase from bovine liver, which is a hexamer with a subunit molecular mass of 27.3 kDa (61), the native enzyme from *C. acetobutylicum* is composed of four identical subunits, each with an approximate molecular mass of 40 kDa and 370 amino acid residues (60). The gene encoding crotonase from *C. difficile* has been incompletely cloned and sequenced (47). This gene is located upstream of the *hbd* gene and is followed by an inverted-repeat termination structure.

BCD is a bacterial analog of short-chain ACD (SACD) from mammalian mitochondria, which catalyzes the α,β desaturation of acyl-CoA substrates (4, 25). ACD generally contains flavin adenine dinucleotide as a cofactor which is tightly bound to the apoenzyme. Moreover, ACD is a tetramer with a subunit molecular mass of 41.7 to 44.5 kDa and a function requiring an electron transfer flavoprotein (ETF) as an electron donor-acceptor (31, 41, 42, 48). In *C. acetobutylicum*, BCD acts in the

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TABLE 1. Bacterial strains, phage, and plasmids employed in this study

Strain, phage, or plasmid	Function(s)	Characteristics[a]	Source or reference
Strains			
C. acetobutylicum ATCC 824	DNA-RNA isolations, electrotransformation		ATCC[b]
E. coli Q358	Phage propagation	$hsdR_k$ $hsdM_k^+$ *supF* $\phi 80^r$	40
E. coli ER2275	Methylation	*recA mcrBC*	New England BioLabs
E. coli XL1-Blue	Subcloning	*hsdR17* (r_k^- r_m^-) (F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10* [Tc^r])	10
Plasmids			
pUC19	Subcloning	ColE1 *ori* Ap^r	69
pSYL2	Shuttle vector	pCBU2 *ori* ColE1 *ori* Tc^r Em^r	37
pAN1	Methylation	p15A *ori* ϕ3T I Cm^r Tc^r	43
pb37	Subcloning of *hbd* and partial *etfA*	Same as pUC19 but crotonase$^-$ BHBD$^+$	This study
pC10	Subcloning of *BCS* operon	Same as pUC19 but crotonase$^+$ BHBD$^+$	This study
pSYL2-BCS	Expression of *BCS* operon	Same as pSYL2 but crotonase$^+$ BCD$^+$ BHBD$^+$	This study
Bacteriophage EMBL3			24

[a] Abbreviations not in the text: *recA*, homologous recombination abolished; *mcrBC*, lacking methycytosine-specific restriction system; Cm^r, Em^r, Tc^r, and Ap^r, chloramphenicol, erythromycin, tetracycline, and ampicillin resistances, respectively; ϕ3T I, ϕ3T methylase.
[b] ATCC, American Type Culture Collection.

reverse direction from fatty acid degradation to produce reduced butyryl-CoA. Characterization of this enzyme from *C. acetobutylicum* has not been reported. However, BCD and the related ETF, which transfers electrons between NAD(H) and BCD, were purified from the butyrate-producing anaerobes *Megasphaera elsdenii* (18, 58) and *Peptostreptococcus elsdenii* (19, 63), and their properties were characterized (20, 50, 65). Moreover, the three-dimensional structure of BCD from *M. elsdenii* has been defined (16) and its encoding gene has been cloned and sequenced (2). Many properties of the bacterial BCD are similar to those of the corresponding enzyme obtained from mammalian mitochondria, and the encoding gene exhibited high-level amino acid similarity to its mammalian counterpart.

Recently, recombinant DNA methods have been used to manipulate specific metabolic pathways in *C. acetobutylicum* (26, 45). A better understanding of the regulatory mechanisms of genes critical to the clostridial metabolic pathways is important in performing successful genetic engineering to enhance product yields. The cloning and expression of genes encoding enzymes in the central fermentation pathway, for example, will provide materials for gene inactivation, enzyme analysis, and progressive metabolic studies. Such studies not only make possible the generation of efficient strains for industrial applications but also provide a better understanding of the functions of metabolic enzymes and the factors controlling their expression. This report describes the successful cloning, sequencing, and expression of clustered genes encoding BHBD, crotonase, BCD, and related ETF enzymes from *C. acetobutylicum*. A possible promoter region for this gene cluster has been identified, which suggests that the genes encoding these enzymes form an operon. In addition, the cloned operon was reintroduced into *C. acetobutylicum*. This study significantly improved the understanding of the genetic structures and other elements in the butyryl-CoA synthesis pathway.

MATERIALS AND METHODS

Bacteria, phage, and growth conditions. The bacterial strains and phage used in this study are listed in Table 1. *C. acetobutylicum* ATCC 824 and *E. coli* were grown and maintained as previously described (26). Media were supplemented with ampicillin (50 μg/ml), erythromycin (250 μg/ml for Luria-Bertani medium and 100 μg/ml for clostridium growth medium [26]), chloramphenicol (32 μg/ml), or tetracycline (10 μg/ml) as required. The titer of the *C. acetobutylicum* phage library was determined and screened with a lambda medium (40) containing a 1.5% bottom agar and a 0.7% top agarose. Phage lysates were collected and stored in a storage medium solution (40) at 4°C.

DNA isolation and manipulation. The plasmids used in this study are also listed in Table 1. Total cellular DNA from *C. acetobutylicum* was prepared as described in reference 11. Construction of a *C. acetobutylicum* genomic library with lambda cloning vector EMBL3 had been previously accomplished (12). Rapid, small-scale plasmid DNA isolation was performed by the alkaline lysis method (40). Large-scale plasmid DNA preparation was done with a Qiagen plasmid purification kit in accordance with the manufacturer's instructions. Phage DNA was prepared by the rapid-plate lysate method as previously described (40). Restriction enzyme-digested genomic, phage, or plasmid fragments were separated by a SeaPlaque low-melting-point agarose (FMC BioProducts) gel, and the fragments of interest were then purified from the gel with GELase (Epicentre Technologies). The resulting DNA fragments were then used for ligation or subcloning.

Probe generation through PCR. Synthetic oligonucleotides, which were 20 to 30 nucleotides long and based on conserved sequences of the known BHBD- or HAD-encoding genes, were designed as primers for PCR analyses with chromosomal ATCC 824 DNA as the template. The reaction mixture and procedure were those recommended by the manufacturer (Perkin Elmer Cetus), except that the final concentration of dATP and dTTP was 250 μM, whereas that of dCTP and dGTP was 150 μM. These adjustments were made because the clostridial DNA is AT rich.

Six sets of primer pairs were designed to generate the necessary BHBD gene-specific screening probe. Of these pairs, the most successful set was generated from nucleotide position 547 to 574 (5′ primer) and from nucleotides complementary to 820 to 847 (3′ primer) (Fig. 2 of reference 70). A DNA fragment of the expected ~300-bp size was amplified and used as the probe for λ phage library screening.

DNA hybridization. The PCR probe was radiolabeled with the random-primer DNA labeling kit (GIBCO BRL) and [α-^{32}P]dATP (3,000 Ci/mmol; ICN). The radiolabeled probe was denatured in a 0.1 N NaOH solution and then purified through a Sephadex G-50 column. Genomic, phage, and plasmid DNAs were digested to completion with restriction enzymes and then separated by agarose gel electrophoresis. The DNA was transferred to Immobilon-N filters (Schleicher & Schuell) in accordance with the manufacturer's instructions. Phage plaques were blotted onto nitrocellulose membranes (Schleicher & Schuell) by the method of Benton and Davis (5). Blotted filters were prehybridized, hybridized, and washed as described previously (12).

Cell transformation. Plasmid transformation to *E. coli* cells was routinely performed with competent cells prepared by the RbCl method of Raleigh (53) or by the method of Chung and Miller (14). Plasmid transformation to *C. acetobutylicum* was performed by the electroporation method (44). Prior to the transformation, plasmids were methylated by transforming the plasmids to *E. coli* ER2275 harboring methylating plasmid pAN1 (Table 1), which expresses the *Bacillus subtilis* phage ϕ3T methytransferase and protects the plasmids from restriction by clostridial endonuclease *Cac*824I (43). The methylated plasmids were then prepared by the Qiagen method and concentrated and desalted with

a Microcon-100 microconcentrator (Amicon) in accordance with the manufacturer's instructions.

Preparation of cell extracts and enzyme assays. Recombinant plasmids were used to transform *E. coli* XL1-Blue or *C. acetobutylicum*. Transformants were grown in 50 ml of either Luria-Bertani or clostridium growth medium supplemented with the corresponding antibiotics. After cells reached the stationary phase, transformants were harvested by centrifugation. For BHBD and crotonase assays, cells were resuspended in 50 mM 4-morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0) containing 1 mM 1,4-dithiothreitol. The cell suspension was sonicated in a W-225R sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) at 60% power for 9 to 15 min at 4°C. Cell debris was removed by centrifugation at 30,000 × *g* for 30 min at 4°C. For BCD assay, cells were resuspended in anaerobic MOPS buffer and 1,4-dithiothreitol was omitted. The cell suspensions were treated with lysozyme and then disrupted by vigorous vortexing for 10 min inside the anaerobic chamber at 0°C. The suspension was capped tightly during centrifugation. After centrifugation, the supernatant was transferred into ampoules and sealed tightly to prevent contact with air.

BHBD activity was analyzed by determining the rate of oxidation of NADH, as measured by the decrease in A_{340} with acetoacetyl-CoA as the substrate (27). Crotonase activity was analyzed by observing the decreasing absorbance of crotonyl-CoA in the specific absorption band at 263 nm (27). BCD activity was analyzed by monitoring the reduction of the ferricenium ion, with an extinction coefficient of 4,300 M^{-1} cm^{-1} (38). All absorbance measurements were conducted on a Gilford model 250 spectrophotometer, and all of the CoA derivatives used for the assays were obtained from Sigma Chemical Company (St. Louis, Mo.). The protein concentration was measured by the dye-binding method of Bradford (8) with bovine serum albumin (Bio-Rad) as the standard.

Protein purification and sequencing. Purified crotonase was obtained as previously described (60), except that the cells were harvested at the late-stationary phase and ruptured by sonication. After purification, samples were loaded onto a sodium dodecyl sulfate–12% polyacrylamide gel and a band in the range of 29 kDa was identified (data not shown). This 29-kDa band was processed for sequencing as previously described (12). The N-terminal amino acid sequencing data were collected by Richard G. Cook, Baylor College of Medicine, Houston, Tex.

DNA sequence analysis. Recombinant plasmid DNA was prepared for sequencing with a Qiagen purification kit. DNA sequencing of both strands was conducted by the dideoxy-chain termination method (55). The DNA was radiolabeled with [^{35}S]dATP (ICN) and primed with oligonucleotides with a Sequenase Version 2.0 sequencing kit (U.S. Biochemicals) as specified by the manufacturer.

RNA isolation and primer extension. Total RNA isolation and primer extension analysis were performed essentially as previously described (59), except that the RNA was isolated from late-stationary-phase cultures. Hybridization of RNA with the radiolabeled primers was performed at room temperature.

Computer programs. Sequence searches were performed with the Genetics Computer Group sequence analysis package, version 8.0. The search programs employed included Blast, tFastA, FastA, Bestfit, Pileup, and Prettyplot.

Nucleotide sequence accession number. The complete nucleotide and amino acid sequences of the *BCS* operon (Fig. 1) have been submitted to GenBank and assigned accession number U17110.

RESULTS

Screening of the phage library. A 300-bp BHBD gene-specific PCR product was used as a probe for λ library screening as described in Materials and Methods. After tertiary screening, positive-phage DNA was isolated. The genomic and positive-phage DNAs were digested with *Hin*dIII, *Eco*RV, and *Bgl*II and then blotted onto membranes for Southern hybridization. Fragments of a 1.3-kb *Hin*dIII-digested phage DNA, a 3.6-kb *Eco*RV-digested phage DNA, and a 9.7-kb *Bgl*II-digested phage DNA were hybridized to the probe; they were the same size as those from the corresponding genomic DNA digests (data not shown). This suggested that the expected chromosomal region containing an *hbd* gene is located on the recombinant phage. This result also suggested that only one copy of the *hbd* gene is present in the chromosome. A restriction map of this region of the *C. acetobutylicum* chromosomal fragment is shown in Fig. 2.

Subcloning, mapping, and sequencing. The 3.6-kb *Eco*RV-digested and 9.7-kb *Bgl*II-digested phage DNA fragments were ligated with *Sma*I- and *Bam*HI-cleaved pUC19 vectors, respectively. The resulting recombinant plasmids were correspondingly designated pb37 and pC10. Various restriction enzymes were used for mapping, and the aligned maps of the *C. acetobutylicum* chromosome and subcloned segments in plasmids pb37 and pC10 are shown in Fig. 2. The 3.6-kb *Eco*RV insert of pb37 is situated within the 9.7-kb *Bgl*II insert of plasmid pC10. Nucleotide sequencing of the inserts for both subclones was initially conducted with the PCR primers previously mentioned. The gene *hbd*, encoding BHBD from *C. acetobutylicum* ATCC 824, with 282 amino acid residues and a molecular mass of 30.5 kDa, was located at the 3′ end of the pC10 insert. The gene was preceded by a putative Shine-Dalgarno site with the sequence AGGGAGG located 8 bp upstream of the ATG start codon. A stem-loop sequence existed 9 bp downstream of the TAA stop codon (Fig. 1).

Further nucleotide sequencing of the region upstream of *hbd* revealed a second open reading frame (ORF), located 143 bp upstream of *hbd*, encoding a protein with 337 amino acids and a molecular mass of 36.1 kDa (Fig. 1). A search of protein sequence data banks revealed that the deduced amino acid sequence encoded by this ORF had a very high degree of homology with those encoded by the *fixB* genes of *C. acetobutylicum* P262 (71), *Azorhizobium caulinodans* (1), and *Rhizobium meliloti* (17) and with the α subunit of human (23), rat (56), and *Paracoccus denitrificans* (3) ETFs. This gene is designated *etfA* and is also preceded by a putative Shine-Dalgarno sequence (AGGAGG), located 9 bp upstream of the methionine start codon.

ETF is a member of the flavoprotein group and plays an important role in the β oxidation of fatty acids and in oxidative demethylation reactions by coupling several flavoprotein dehydrogenases to the electron transport chain (28, 62). ETFs or ETF-like proteins have been isolated from both mammalian sources and a variety of bacteria, such as *P. elsdenii* (63), *M. elsdenii* (50), and *P. denitrificans* (29, 62). Most of the ETFs identified and characterized so far are heterodimers consisting of α and β subunits (50, 62, 63).

The nucleotide sequence of *etfA* from *C. acetobutylicum* ATCC 824 also shows high-level similarity to the *fixB* gene, which was originally identified within the *fixABCX* operon from different rhizobia and nonsymbiotic bacteria such as *A. caulinodans* (1). Three ORFs of this operon, *fixA*, *fixB*, and *fixC*, encode hypothetical proteins with sequence similarity to the β and α subunits of ETF and ETF-ubiquinone oxidoreductase, respectively (1, 3). No specific biochemical function has been identified for the *fixABCX* gene products, but it has been suggested that such products are involved in electron transport to nitrogenase (1).

A third ORF, encoding 252 amino acid codons (27.2 kDa), was found 37 bp upstream of *etfA* (Fig. 1). A computer search of the deduced peptide sequence demonstrated direct similarities between this gene product and the β subunit of human (22) and *P. denitrificans* (3) ETFs and between this same product and those of the *fixA* genes of *A. caulinodans* (1) and *R. meliloti* (17). This gene, designated *etfB*, is preceded by the putative Shine-Dalgarno sequence AGGAGG and an 8-bp space.

A fourth ORF, of 379 residues, was located 17 bp upstream of *etfB* (Fig. 1), and a computer search revealed high-level similarity between this gene product and those of various ACDs (31, 41, 42, 48). Because of the nature of BCD, which reacts with its related ETF, this ORF most probably represents the structural gene that encodes BCD and is therefore designated *bcd*. The putative Shine-Dalgarno sequence AGGAGG is located 9 bp upstream of the *bcd* Met start codon.

A fifth ORF, encoding a polypeptide with 261 residues and a molecular mass of 28.2 kDa, was located 13 bp upstream of *bcd*. The sequencing data for this ORF show homology with the incompletely cloned crotonase gene from *C. difficile* (47)

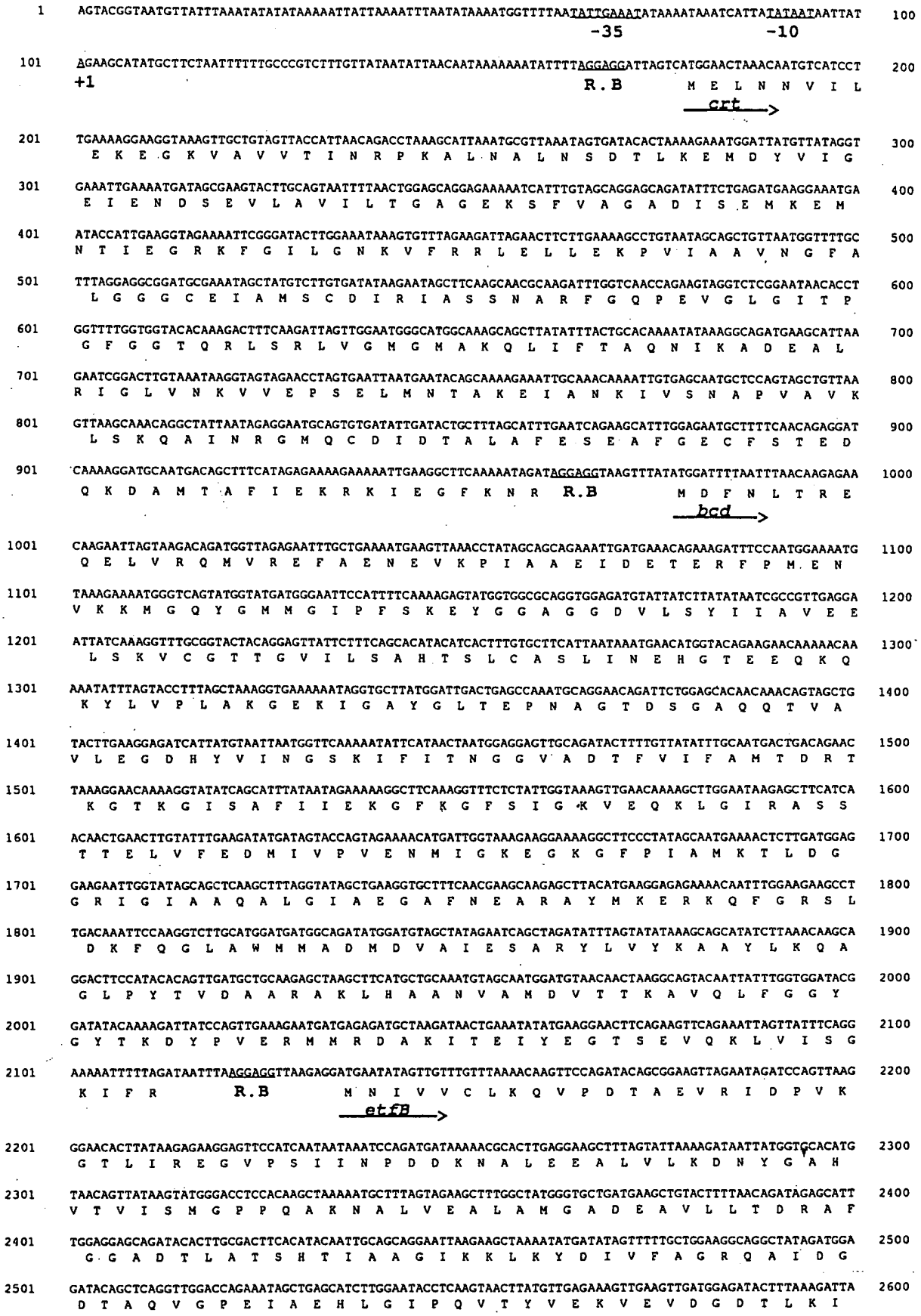

FIG. 1. Nucleotide and deduced amino acid sequences of the genes in the *BCS* operon. The standard one-letter amino acid abbreviation is shown under the second nucleotide of each codon. The putative ribosome-binding (R.B.) site, the promoter sequence −35 and −10 regions, and the transcriptional start site (+1) are underlined. A potential transcriptional terminator is indicated by convergent arrows. The putative NAD-binding site for the *hbd* gene is marked with asterisks.

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2601  GAAAAGCTTGGGAAGATGGATATGAAGTTGTTGAAGTTAAGACACCAGTTCTTTTAACAGCAATTAAAGAATTAAATGTTCCAAGATATATGAGTGTAGA  2700
      R  K  A  W  E  D  G  Y  E  V  V  E  V  K  T  P  V  L  L  T  A  I  K  E  L  N  V  P  R  Y  M  S  V  E

2701  AAAAATATTCGGAGCATTTGATAAAGAAGTAAAAATGTGGACTGCCGATGATATAGATGTAGATAAGGCTAATTTAGGTCTTAAAGGTTCACCAACTAAA  2800
        K  I  F  G  A  F  D  K  E  V  K  M  W  T  A  D  D  I  D  V  D  K  A  N  L  G  L  K  G  S  P  T  K

2801  GTTAAGAAGTCATCAACTAAAGAAGTTAAAGGACAGGGAGAAGTTATTGATAAGCCTGTTAAGGAAGCAGCTGATATGTTGTCTCAAAATTAAAAGAAGA  2900
       V  K  K  S  S  T  K  E  V  K  G  Q  G  E  V  I  D  K  P  V  K  E  A  A  D  M  L  S  Q  N

2901  ACACATATTTAAGTTAGGAGGGATTTTTCAATGAATAAAGCAGATTACAAGGGCGTATGGGTGTTTGCTGAACAAAGAGACGGAGAATTACAAAAGGTAT  3000
                     R.B.           M  N  K  A  D  Y  K  G  V  W  V  F  A  E  Q  R  D  G  E  L  Q  K  V
                                   ____etfA____>

3001  CATTGGAATTATTAGGTAAAGGTAAGGAAATGGCTGAGAAATTAGGCGTTGAATTAACAGCTGTTTTACTTGGACATAATACTGAAAAAATGTCAAAGGA  3100
      S  L  E  L  L  G  K  G  K  E  M  A  E  K  L  G  V  E  L  T  A  V  L  L  G  H  N  T  E  K  M  S  K  D

3101  TTTATTATCTCATGGAGCAGATAAGGTTTTAGCAGCAGATAATGAACTTTTAGCACATTTTTCAACAGATGGATATGCTAAAGTTATATGTGATTTAGTT  3200
        L  L  S  H  G  A  D  K  V  L  A  A  D  N  E  L  L  A  H  F  S  T  D  G  Y  A  K  V  I  C  D  L  V

3201  AATGAAAGAAAGCCAGAAATATTATTCATAGGAGCTACTTTCATAGGAAGAGATTTAGGACCAAGAATAGCAGCAAGACTTTCTACTGGTTTAACTGCTG  3300
       N  E  R  K  P  E  I  L  F  I  G  A  T  F  I  G  R  D  L  G  P  R  I  A  A  R  L  S  T  G  L  T  A

3301  ATTGTACATCACTTGACATAGATGTAGAAAATAGAGATTTATTGGCTACAAGACCAGCGTTTGGTGGAAATTTGATAGCTACAATAGTTTGTTCAGACCA  3400
      D  C  T  S  L  D  I  D  V  E  N  R  D  L  L  A  T  R  P  A  F  G  G  N  L  I  A  T  I  V  C  S  D  H

3401  CAGACCACAAATGGCTACAGTAAGACCTGGTGTGTTTTTTGAAAAATTACCTGTTAATGATGCAAATGTTTCTGATGATAAAATAGAAAAAGTTGCAATT  3500
        R  P  Q  M  A  T  V  R  P  G  V  F  F  E  K  L  P  V  N  D  A  N  V  S  D  D  K  I  E  K  V  A  I

3501  AAATTAACAGCATCAGACATAAGAACAAAAGTTTCAAAAGTTGTTAAGCTTGCTAAAGATATTGCAGATATCGGAGAAGCTAAGGTATTAGTTGCTGGTG  3600
       K  L  T  A  S  D  I  R  T  K  V  S  K  V  V  K  L  A  K  D  I  A  D  I  G  E  A  K  V  L  V  A  G

3601  GTAGAGGAGTTGGAAGCAAAGAAAACTTTGAAAAACTTGAAGAGTTAGCAAGTTTACTTGGTGGAACAATAGCCGCTTCAAGAGCAGCAATAGAAAAAGA  3700
      G  R  G  V  G  S  K  E  N  F  E  K  L  E  E  L  A  S  L  L  G  G  T  I  A  A  S  R  A  A  I  E  K  E

3701  ATGGGTTGATAAGGACCTTCAAGTAGGTCAAACTGGTAAAACTGTAAGACCAACTCTTTATATTGCATGTGGTATATCAGGAGCTATCCAGCATTTAGCA  3800
        W  V  D  K  D  L  Q  V  G  Q  T  G  K  T  V  R  P  T  L  Y  I  A  C  G  I  S  G  A  I  Q  H  L  A

3801  GGTATGCAAGATTCAGATTACATAATTGCTATAAATAAAGATGTAGAAGCCCCAATAATGAAGGTAGCAGATTTGGCTATAGTTGGTGATGTAAATAAAG  3900
       G  M  Q  D  S  D  Y  I  I  A  I  N  K  D  V  E  A  P  I  M  K  V  A  D  L  A  I  V  G  D  V  N  K

3901  TTGTACCAGAATTAATAGCTCAAGTTAAAGCTGCTAATAATTAAGATAAATAAAAAGAATTATTTAAAGCTTATTATGCCAAAATACTTATATAGTATTT  4000
      V  V  P  E  L  I  A  Q  V  K  A  A  N  N
                                                                                                     *  *
4001  TGGTGTAAATGCATTGATAGTTTCTTTAAATTTAGGGAGGTCTGTTTAATGCATTGATAGTTCTTTAAATTTAGGGAGGTCTGTTTAATGAAAAAGGTAT  4100
                                                                                       R.B.     M  K  K  V
                                                                                              ____hbd____>
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
4101  GTGTTATAGGTGCAGGTACTATGGGTTCAGGAATTGCTCAGGCATTTGCAGCTAAAGGATTTGAAGTAGTATTAAGAGATATTAAAGATGAATTTGTTGA  4200
      C  V  I  G  A  G  T  M  G  S  G  I  A  Q  A  F  A  A  K  G  F  E  V  V  L  R  D  I  K  D  E  F  V  D

4201  TAGAGGATTAGATTTTATCAATAAAAATCTTTCTAAATTAGTTAAAAAAGGAAAGATAGAAGAAGCTACTAAAGTTGAAATCTTAACTAGAATTTCCGGA  4300
        R  G  L  D  F  I  N  K  N  L  S  K  L  V  K  K  G  K  I  E  E  A  T  K  V  E  I  L  T  R  I  S  G

4301  ACAGTTGACCTTAATATGGCAGCTGATTGCGATTTAGTTATAGAAGCAGCTGTTGAAAGAATGGATATTAAAAAGCAGATTTTTGCTGACTTAGACAATA  4400
        T  V  D  L  N  M  A  A  D  C  D  L  V  I  E  A  A  V  E  R  M  D  I  K  K  Q  I  F  A  D  L  D  N

4401  TATGCAAGCCAGAAACAATTCTTGCATCAAATACATCATCACTTTCAATAACAGAAGTGGCATCAGCAACTAAAACTAATGATAAGGTTATAGGTATGCA  4500
      I  C  K  P  E  T  I  L  A  S  N  T  S  S  L  S  I  T  E  V  A  S  A  T  K  T  N  D  K  V  I  G  M  H

4501  TTTCTTTAATCCAGCTCCTGTTATGAAGCTTGTAGAGGTAATAAGAGGAATAGCTACATCACAAGAAACTTTTGATGCAGTTAAAGAGACATCTATAGCA  4600
        F  F  N  P  A  P  V  M  K  L  V  E  V  I  R  G  I  A  T  S  Q  E  T  F  D  A  V  K  E  T  S  I  A

4601  ATAGGAAAAGATCCTGTAGAAGTAGCAGAAGCACCAGGATTTGTTGTAAATAGAATATTAATACCAATGATTAATGAAGCAGTTGGTATATTAGCAGAAG  4700
       I  G  K  D  P  V  E  V  A  E  A  P  G  F  V  V  N  R  I  L  I  P  M  I  N  E  A  V  G  I  L  A  E

4701  GAATAGCTTCAGTAGAAGACATAGATAAAGCTATGAAACTTGGAGCTAATCACCCAATGGGACCATTAGAATTAGGTGATTTTATAGGTCTTGATATATG  4800
      G  I  A  S  V  E  D  I  D  K  A  M  K  L  G  A  N  H  P  M  G  P  L  E  L  G  D  F  I  G  L  D  I  C

4801  TCTTGCTATAATGGATGTTTTATACTCAGAAACTGGAGATTCTAAGTATAGACCACATACATTACTTAAGAAGTATGTAAGAGCAGGATGGCTTGGAAGA  4900
        L  A  I  M  D  V  L  Y  S  E  T  G  D  S  K  Y  R  P  H  T  L  L  K  K  Y  V  R  A  G  W  L  G  R

4901  AAATCAGGAAAAGGTTTCTACGATTATTCAAAATAAGTTTACAAGAATCCCCATTATCAAATGGGGATTTTTTATATATAATATAATTTTAGA

        K  S  G  K  G  F  Y  D  Y  S  K                ------->     <-------
```

FIG. 1—*Continued.*

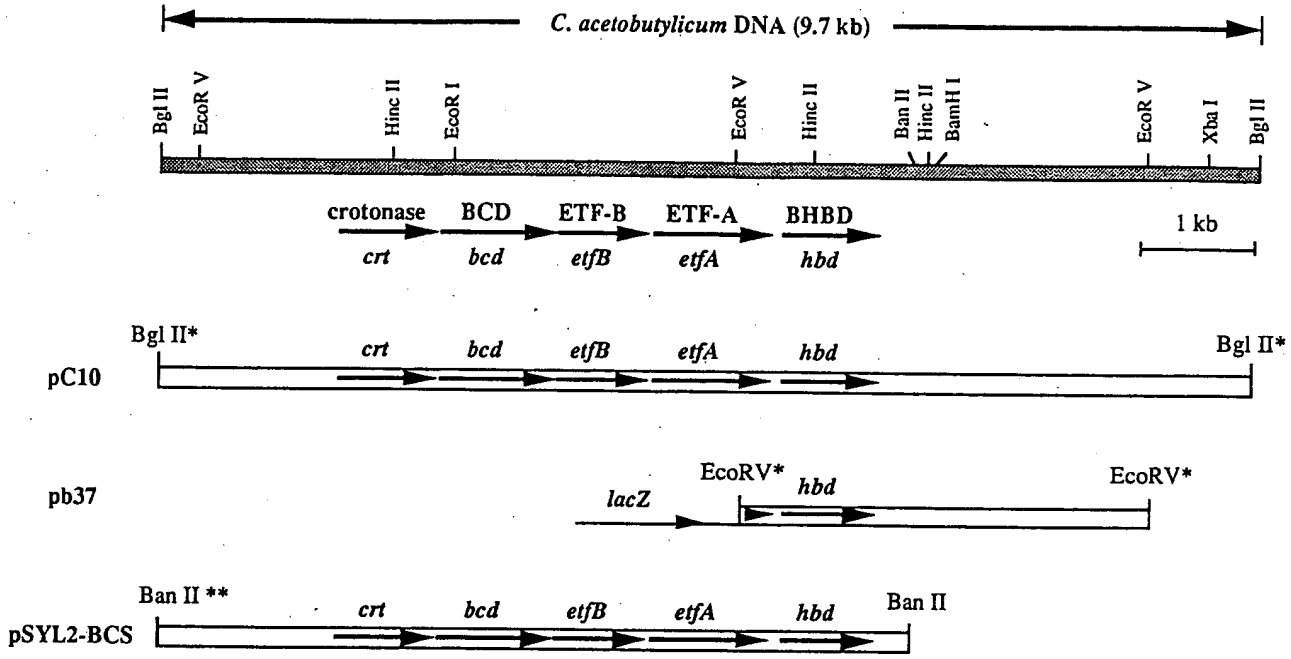

FIG. 2. Transcriptional organization within the clustered genes encoding the enzymes involved in butyryl-CoA synthesis. The restriction sites of the inserts for plasmids pb37, pC10, and pSYL2-BCS are shown. An asterisk indicates loss of the *Bgl*II sites for the pC10 insert and of the *Eco*RV sites for the pb37 insert because of ligation with *Bam*HI- and *Sma*I-digested pUC19 vectors, respectively. The 3.6-kb *Eco*RV insert of pb37 is located between the second and third, from the left, *Eco*RV sites and contains the partial ETF-A and the entire BHBD coding sequences. Two asterisks indicate that the *Ban*II site is derived from the polylinker cloning site of pUC19.

and with rat ECH (46), *Caenorhabditis elegans* ECH (66), and the ECH part of the fatty oxidation complex of *E. coli* (67). Therefore, this ORF appears to be that for the *crt* gene, encoding a 28.2-kDa crotonase from *C. acetobutylicum* ATCC 824.

Further sequencing up to a point 500 bp upstream of *crt* and 1.2 kb downstream of *hbd* revealed no obvious ORFs which would encode metabolically related enzymes of the clostridial pathways. No transcriptional termination sequences were found in the intergenic regions between the five genes cloned in this study.

Primer extension. Primer extension experiments were conducted with primers homologous to the N-terminal nucleotide sequences of the *crt* gene, and one single transcriptional start site (Fig. 3) was determined and located 77 bp upstream of the *crt* start codon. The corresponding −10 and −35 regions for this site matched closely with the consensus promoter sequences. No other transcriptional start sites were identified within 150 bp of the start codon of all remaining genes through primer extension experiments, suggesting that the clustered genes were transcribed as a single unit to form an operon, designated *BCS* (butyryl-CoA synthesis). The complete nucleotide and amino acid sequences of this operon are summarized in Fig. 1.

Enzyme activity and expression. BHBD, crotonase, and BCD assays were performed with crude extracts from *E. coli* XL1-Blue containing either pUC19 (control), pb37, or pC10. These enzyme activities are shown in Table 2. *E. coli* cells harboring pb37 exhibited an NADH-dependent BHBD activity approximately 60 times greater than that of the control, whereas *E. coli* cells harboring pC10 exhibited BHBD activity 70 times greater and crotonase activity about 140 times higher than that of the control strain.

The assay for BCD activity in *E. coli* was not successful,

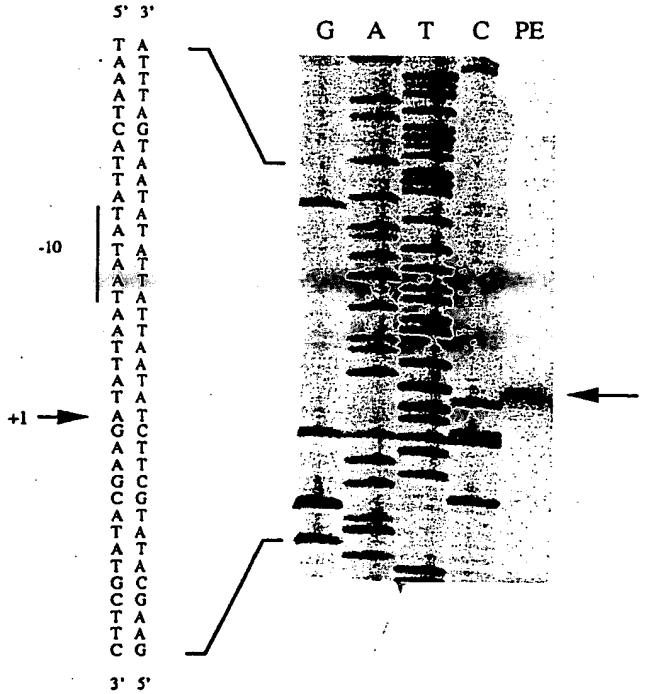

FIG. 3. Determination of the transcription start site by primer extension analysis. An oligonucleotide complementary to the *crt* sequence (from nucleotide position 217 to 233 [Fig. 1]) was hybridized to RNA isolated from a stationary-phase culture of *C. acetobutylicum* and extended by reverse transcriptase. Lane PE represents the primer extension product. Lanes G, A, T, and C represent the sequencing reaction products obtained by using the same oligonucleotide as the primer and recombinant plasmid pC10 as the template.

TABLE 2. Enzyme activities of recombinant *E. coli* and *C. acetobutylicum* strains

Organism and plasmid	Mean sp act (U/mg)[a]		
	Crotonase	BHBD	BCD
Aerobically grown *E. coli*			
pUC19	0.1	0.1	—[b]
pb37	0.1	5.6	—
pC10	13.7	7.4	—
pSYL2	0.3	0.1	—
pSYL2-BCS	46.5	11.6	—
Anaerobically grown *E. coli*			
pSYL2	0.1	<0.03	—
pSYL2-BCS	187.5	8.6	—
C. acetobutylicum			
pSYL2	67.0	6.6	1.2
pSYL2-BCS	128.6	11.0	3.3

[a] Data shown are based on at least two independently prepared extracts and were reproducible within 15% of the value given. One unit is the amount of enzyme that converts 1 μmol of the substrate to the product per min.

[b] —, undetectable; the minimum measurable specific activity of BCD is ~0.04 U/mg.

probably because of the instability of this enzyme. A 6.8-kb *Ban*II fragment containing the entire operon was therefore subcloned to *E. coli-C. acetobutylicum* shuttle vector pSYL2, which contains both *E. coli* and clostridial origins of replication (36, 37). The subclone was designated pSYL2-BCS. Both pSYL2 and pSYL2-BCS were used to transform either *E. coli* XL1-Blue or *C. acetobutylicum* as described in Materials and Methods. Crude extracts from cultures containing either pSYL2 (control) or pSYL2-BCS were assayed for BHBD, crotonase, and BCD activities, and the results are shown in Table 2. During the stationary phase, the BHBD and crotonase activities were elevated significantly in *E. coli* harboring plasmid pSYL2-BCS. However, BCD activity was undetected in *E. coli* extracts made from cells grown either aerobically or anaerobically. For *C. acetobutylicum*, the BHBD, crotonase, and BCD activity levels increased two- to threefold when the cells harbored the subclone (Table 2).

Protein sequence analysis and alignment. The N-terminal amino acid sequence of the purified 29-kDa crotonase was determined, and the first 10 amino acid residues were as follows: Lys/Thr-Glu-Leu-Asn-Asn-Val-Ile-Leu-Glu-Lys. Purified crotonase exhibited a molecular size much smaller than that previously reported (29 versus 43 kDa) (60). However, the N-terminal sequencing data from the purified crotonase agree closely with the peptide sequence deduced from its cloned gene, except that the first methionine residue from the N-terminal sequencing data was ambiguous. The reason for the somewhat smaller crotonase molecular size is not known. However, this subunit is quite similar in size to the corresponding purified bovine enzyme (61).

The best alignment of the *C. acetobutylicum* crotonase sequence demonstrated the following identities and similarities (in parentheses) with the related proteins shown: 64% (79%) in a 155-amino-acid overlap for *C. difficile*, 41% (66%) for rats, 40% (65%) for *C. elegans*, and 34% (59%) for *E. coli*. The corresponding BCD values are 56% (73%) for *M. elsdenii*, 48% (68%) for rats, and 48% (66%) for humans. The corresponding ETF-B values are 46% (67%) for *R. meliloti*, 46% (66%) for *A. caulinodans*, 32% (55%) for *P. denitrificans*, and 30% (56%) for humans. The corresponding ETF-A values are 74% (86%) for *C. acetobutylicum* P262, 45% (67%) for *A. caulinodans*, 46% (65%) for *R. meliloti*, 39% (61%) for humans, 36% (58%) for *P. denitrificans*, and 35% (59%) for rats.

The amino acid sequence deduced from *hbd* contains 282 residues, the same as that from *C. acetobutylicum* P262 (70). In fact, they had 87% similarity and 78% identity. The *hbd* gene from *C. acetobutylicum* ATCC 824 also has 75% similarity and 64% identity with *hbd* from *C. difficile* (47), 61% similarity and 44% identity with the equivalent porcine HAD from fatty acid β oxidation (7), and 56% similarity and 38% identity with the HAD portion of the bifunctional enzyme from rat peroxisomes (49). The *hbd* gene also exhibited 55% similarity and 37% identity with the HAD portion of the *fadBA* operon which encodes a multifunctional enzyme complex in *E. coli* (57, 68).

DISCUSSION

In the central acetone-butanol fermentation pathway, BHBD, crotonase, and BCD catalyze the conversion of acetoacetyl-CoA to butyryl-CoA. There have been no reports of the purification and characterization of all of the enzymes responsible for this conversion. The cloning of the genes encoding these enzymes has therefore provided new information concerning gene arrangement and regulation. It has been shown that the specific activity levels of BHBD and crotonase in *C. acetobutylicum* follow similar patterns throughout the course of fermentation (27). Judging from the gene arrangement (Fig. 2), it is probable that these genes form an operon which corresponds to a regulatory unit of the associated functions. This suggestion was further confirmed through primer extension experiments (Fig. 3). A single transcriptional start site was identified upstream of *crt*, but none was identified upstream of any other gene in the cluster.

The *crt* and *hbd* genes are highly expressed in *E. coli* (Table 2). However, BCD activity was detected only in *C. acetobutylicum* extract and not in *E. coli* extract, even when *E. coli* was grown under anaerobic conditions. The reason for this finding is not known, but it may result from improper folding of the expressed protein and its lack of function in *E. coli*. Although BHBD, crotonase, and BCD activities are elevated in *C. acetobutylicum* harboring pSYL2-BCS, the elevated levels are not as high as for those in *E. coli*. It is possible that the specific activity levels of BHBD, crotonase, and BCD in *C. acetobutylicum* are already very high (27, 60). An increased gene dosage might not exhibit as dramatic an effect on enzyme activity levels as in *E. coli*, where the basal activity of BHBD, crotonase, or BCD is at a very low level (Table 2). Alternatively, perhaps an autoregulatory control exists.

The cloning of the *etfAB* genes provides insight into important but previously unidentified elements in the clostridial butyryl-CoA synthesis pathway. Neither ETF nor the related enzyme BCD has been purified from *C. acetobutylicum*. In fact, it was not until recently that the presence of a BCD-related ETF in solvent-producing clostridia was suggested (13). This presence was suggested primarily because of the isolation of NAD(P)H-specific ETFs from other anaerobic bacteria or mitochondria (15, 35, 50, 63). Previous studies show striking similarities in electrochemical behavior and sequence structure between mammalian and bacterial BCD or ETF (62). Because of these homologies, structural and mechanistic similarities would be expected. Interestingly, the nucleotide sequence of *etfA* also exhibited close homology with that of the *fixB* gene located upstream of *hbd* in *C. acetobutylicum* P262 (71). Judging from the gene structure and arrangement, it is probable that the two are homologous genes from different clostridial strains.

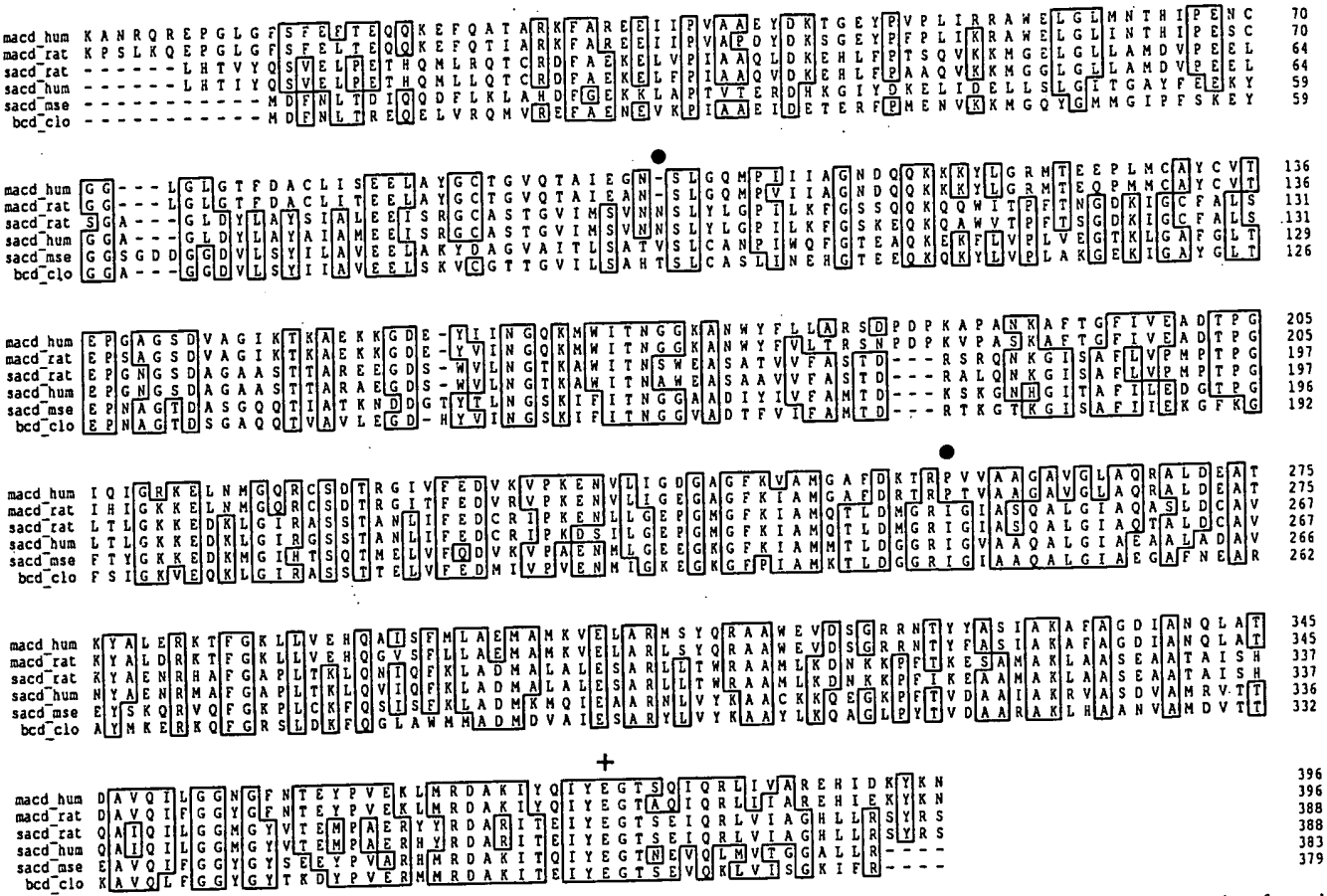

FIG. 4. Alignment of the amino acid sequence of *C. acetobutylicum* BCD and those of functionally related enzymes. The amino acid sequence numbers for each enzyme are shown on the right. Abbreviations: macd_hum and macd_rat, human and rat MACDs, respectively; sacd_rat, sacd_hum, and sacd_mse, rat, human, and *M. elsdenii* SACDs, respectively; bcd_clo, BCD from *C. acetobutylicum* ATCC 824. The Glu residue suggested to be involved in catalysis is marked with a plus sign. The residues suggested to be involved in short-chain specificity are marked with dots.

There are two reasons for the gene designation *etfAB* (*C. acetobutylicum* ATCC 824) instead of *fixBA* (*C. acetobutylicum* P262 [71]). First, ETF from *P. denitrificans* has been purified and characterized (29, 62) and its encoding gene has been cloned and expressed (3). BCD-related ETF has also been purified from the anaerobic bacteria *M. elsdenii* and *P. elsdenii* and characterized (50, 63). The *etfAB* genes from *P. denitrificans* show close homology to the *etfAB* genes from *C. acetobutylicum* ATCC 824. Second, judging from the arrangements of the *etfAB* and *bcd* gene structures of *C. acetobutylicum* ATCC 824 (Fig. 2), it is reasonable to propose that ETF-A and ETF-B form the two subunits of an ETF-like heterodimer. This heterodimer is proposed to be involved in electron transfer to BCD, just as for the mitochondrial α- and β-ETF functions. Finally, the *fixAB* designation for *C. acetobutylicum* P262 was based on sequence homology (71) without the knowledge of the location of the *bcd* structural gene and experimental confirmation of the enzyme functions. Therefore, *etfAB* may be a better designation for these genes and provide a more succinct explanation of the functions of the enzymes they encode.

Various dehydrogenases are characterized by two major domains. One domain is involved in coenzyme binding, and the other is involved in substrate binding (54). The common structure of the NAD-binding site in many dehydrogenases is a βαβ fold centered around a conserved G-x-G-x-x-G sequence (64). A similar supersecondary structure, including residues 3 to 33 (Fig. 1), was observed by alignment of BHBD from strain ATCC 824 with the pig and rat HAD enzymes (data not shown). This observation is in agreement with an X-ray crystallographic study of crystallized porcine HAD at 2.8 Å (0.28 nm) which suggests that the NAD-binding site of this enzyme is located within the amino-terminal domain (6).

Alignment of the deduced amino acid sequence of BCD with those of functionally related enzymes (Fig. 4) showed that in *C. acetobutylicum*, Glu-363 is the residue homologous to Glu-376 in medium-chain ACD (MACD), Glu-368 in SACD, and Glu-367 in BCD from *M. elsdenii*. Recently, the catalytic role of these glutamic acid residues has been confirmed by both site-directed mutagenesis and crystal structure analysis of MACD (9, 33, 34, 51), SACD (32, 39), and BCD from *M. elsdenii* (2, 16). These catalytic glutamic acids are responsible for the α,β-dehydrogenation reaction, and more specifically, the α-proton abstraction (21). Since in *C. acetobutylicum*, Glu-363 is the residue homologous to the noted catalytic sites (Fig. 4), it is highly probable that this residue is active in such catalysis.

The acyl-CoA substrate binding sites of BCD from *M. elsdenii* and porcine MACD have already been carefully demonstrated (16, 33). The reason for substrate specificity between MACD and BCD might be the binding cavity for a different substrate molecule length (16). The relatively shallow cavity for BCD from *M. elsdenii* results from the insertion of a single amino acid, Val-94. This insertion distorts the helix that binds

the substrate, thereby bringing it closer. Another reason for the deeper cavity for MACD is the side chain of residue Pro-257, which results in a helix that is less aligned and farther removed from the substrate. From the alignment data (Fig. 4), it is evident that BCD from *C. acetobutylicum* also contains an additional residue (Thr-91) at the same position as observed in BCD from *M. elsdenii*. In addition, the proline in MACD is replaced by isoleucine in various SACDs, including Ile-244 in *C. acetobutylicum*. It is therefore expected that BCD from *C. acetobutylicum* might also exhibit a shallow substrate-binding cavity, thus explaining the short-chain specificity of this enzyme.

The amino acid sequence alignment of ACDs from rat, human, and bacterial sources (Fig. 4) showed no region that has the clear homology to the flavin adenine dinucleotide-binding sites (GxGxxG patches) found in the other flavin adenine dinucleotide-binding enzymes. This observation is consistent with the results of three-dimensional studies which showed no classical nucleotide-binding domain (βαβ secondary structure) in the vicinity of the flavin adenine dinucleotide-binding site (34). This finding is also consistent with the knowledge that ACDs require an ETF as the electron donor-acceptor, while other flavoproteins contain a binding site for NAD or NADP.

In *C. acetobutylicum* P262, *hbd* is downstream of *fixB* and upstream of *adh-1*, an alcohol dehydrogenase gene. There was no transcription termination sequence between any two of these three genes (70, 71). In *C. difficile*, *hbd* is downstream of a putative crotonase gene and upstream of a thiolase gene, with a termination sequence between the crotonase and *hbd* genes but no such sequence between *hbd* and the gene for thiolase (47). In *C. acetobutylicum* ATCC 824, *hbd* is downstream of *etfA* and is followed by a stem-loop structure after its stop codon (Fig. 1). Sequencing data for the 1.2-kb region downstream of *hbd* revealed no obvious ORF that could correspond to an alcohol dehydrogenase- or thiolase-encoding gene.

It is interesting that the peptide sequences deduced from the *BCS* operon genes all have close homology with the equivalent enzymes involved in fatty acid β oxidation. This characteristic suggests that a common origin and mechanism exist for the fatty acid β-oxidation pathway of mammalian mitochondria and the butanol-butyrate synthesis pathway of clostridia.

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REFERENCES

1. **Arigoni, F., P. A. Kaminski, H. Hennecke, and C. Elmerich.** 1991. Nucleotide sequence of the *fixABC* region of *Azorhizobium caulinodans* ORS571: similarity of the *fixB* product with eukaryotic flavoproteins, characterization of *fixX* and identification of *nifW*. Mol. Gen. Genet. 225:514–520.
2. **Becker, D. F., J. A. Fuchs, D. K. Banfield, W. D. Funk, R. T. A. MacGillivray, and M. T. Stankovich.** 1993. Characterization of wild-type and an active-site mutant in *Escherichia coli* of short-chain acyl-CoA dehydrogenase from *Megasphaera elsdenii*. Biochemistry 32:10736–10742.
3. **Bedzyk, L. A., K. W. Escudero, R. E. Gill, K. J. Griffin, and F. E. Frerman.** 1993. Cloning, sequencing, and expression of the genes encoding subunits of *Paracoccus denitrificans* electron transfer flavoprotein. J. Biol. Chem. 268: 20211–20217.
4. **Beinert, H.** 1963. Acyl coenzyme A dehydrogenases, p. 447–466. *In* P. D. Boyer, H. Lardy, and K. Myrback (ed.), The enzymes, 2nd ed. Academic Press, Inc., New York.
5. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques *in situ*. Science 196:180–182.
6. **Birktoft, J. J., H. M. Holden, R. Hamlin, N. H. Xuong, and L. J. Banaszak.** 1987. Structure of L-3-hydroxyacyl-coenzyme A dehydrogenase: preliminary chain tracing at 2.8-Å resolution. Proc. Natl. Acad. Sci. USA 84:8262–8266.
7. **Bitar, K. G., A. Pepez-Aranda, and R. A. Bradshaw.** 1980. Amino acid sequence of L-3-hydroxyacyl-CoA dehydrogenase from pig heart muscle. FEBS Lett. 116:196–198.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
9. **Bross, P., S. Engst, A. W. Strauss, D. P. Kelly, I. Rasched, and S. Ghisla.** 1990. Characterization of wild-type and an active mutant of human medium chain acyl-CoA dehydrogenase after expression in *Escherichia coli*. J. Biol. Chem. 265:7116–7119.
10. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL-1 Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. Biotechniques 5:376–379.
11. **Cary, J. W., D. J. Petersen, E. T. Papoutsakis, and G. N. Bennett.** 1988. Cloning and expression of *Clostridium acetobutylicum* phosphotransbutyrylase and butyrate kinase genes in *Escherichia coli*. J. Bacteriol. 170:4613–4618.
12. **Cary, J. W., D. J. Petersen, E. T. Papoutsakis, and G. N. Bennett.** 1990. Cloning and expression of *Clostridium acetobutylicum* ATCC 824 acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A-transferase in *Escherichia coli*. Appl. Environ. Microbiol. 56:1576–1583.
13. **Chen, J.-S.** 1993. Properties of acid- and solvent-forming enzymes of clostridia, p. 54–60. *In* D. R. Woods (ed.), The clostridia and biotechnology. Butterworth-Heinemann, Stoneham, Mass.
14. **Chung, C. T., and R. H. Miller.** 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. 16:3580.
15. **Dietrichs, D., M. Meyer, B. Schmidt, and J. R. Andreesen.** 1990. Purification of NADPH-dependent electron-transferring flavoproteins and N-terminal protein sequence data of dihydrolipoamide dehydrogenases from anaerobic, glycine-utilizing bacteria. J. Bacteriol. 172:2088–2095.
16. **Djordjevic, S., C. P. Pace, M. T. Stankovich, and J.-J. P. Kim.** 1995. Three-dimensional structure of butyryl-CoA dehydrogenase from *Megasphaera elsdenii*. Biochemistry 34:2163–2171.
17. **Earl, C. D., C. W. Ronson, and F. M. Ausubel.** 1987. Genetic and structural analysis of the *Rhizobium meliloti fixA*, *fixB*, *fixC*, and *fixX* genes. J. Bacteriol. 169:1127–1136.
18. **Engel, P. C.** 1981. Butyryl-CoA dehydrogenase from *Megasphaera elsdenii*. Methods Enzymol. 71:359–366.
19. **Engel, P. C., and V. Massey.** 1971. The purification and properties of butyryl-coenzyme A dehydrogenase from *Peptostreptococcus elsdenii*. Biochem. J. 125:879–887.
20. **Engel, P. C., and V. Massey.** 1971. Green butyryl-coenzyme A dehydrogenase, an enzyme-acyl-coenzyme A complex. Biochem. J. 125:889–902.
21. **Fendrich, G., and R. H. Abeles.** 1982. Mechanism of action of butyryl-CoA dehydrogenase: reactions with acetylenic, olefinic, and fluorinated substrate analogues. Biochemistry 21:6685–6695.
22. **Finocchiaro, G., I. Colombo, L. Garavaglia, C. Gellera, G. Valdameri, N. Garbuglio, and S. Didonato.** 1993. cDNA cloning and mitochondrial import of the β-subunit of the human electron-transfer flavoprotein. Eur. J. Biochem. 213:1003–1008.
23. **Finocchiaro, G., M. Ito, Y. Iketa, and K. Tanaka.** 1988. Molecular cloning and nucleotide sequence of cDNAs encoding the α-subunit of human electron transfer flavoprotein. J. Biol. Chem. 263:15773–15780.
24. **Frischauf, A.-M., H. Lehroch, A. Poustka, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. 170:827–842.
25. **Green, D. E., S. Mii, R. Mahler, and R. M. Rock.** 1954. Studies on the fatty acid oxidation system of animal tissues. III. Butyryl-CoA dehydrogenase. J. Biol. Chem. 206:1.
26. **Green, E. M., and G. N. Bennett.** Inactivation of an aldehyde/alcohol dehydrogenase gene from *Clostridium acetobutylicum* ATCC 824. Appl. Biochem. Biotech., in press.
27. **Hartmanis, M. G., and S. Gatenbeck.** 1984. Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate. Appl. Environ. Microbiol. 47:1277–1283.
28. **Husain, M., and D. J. Steenkamp.** 1983. Electron transfer flavoprotein from pig liver mitochondria: a simple purification and re-evaluation of some of the molecular properties. Biochem. J. 209:541–545.
29. **Husain, M., and D. J. Steenkamp.** 1985. Partial purification and characterization of glutaryl-coenzyme A dehydrogenase, electron transfer flavoprotein, and electron transfer flavoprotein-Q oxidoreductase from *Paracoccus denitrificans*. J. Bacteriol. 163:709–715.
30. **Jones, D. T., and D. R. Woods.** 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50:484–524.
31. **Kelly, D. P., J. J. Kim, J. J. Billadello, B. E. Hainline, T. W. Chu, and A. W. Strauss.** 1987. Nucleotide sequence of medium-chain acyl-CoA dehydrogenase mRNA and its expression in enzyme-deficient human tissue. Proc. Natl. Acad. Sci. USA 84:4068–4072.
32. **Kim, J.-J. P., M. Wang, S. Djordjevic, R. Paschke, and D. W. Bennett.** 1994.

Three dimensional structures of acyl-CoA dehydrogenases: structure basis of substrate specificity, p. 273–282. *In* K. Yagi (ed.), Flavins and flavoproteins 1993. Walter de Gruyter, Berlin.

33. **Kim, J.-J. P., M. Wang, and R. Paschke.** 1993. Crystal structures of medium-chain acyl-CoA dehydrogenase from pig liver mitochondria with and without substrate. Proc. Natl. Acad. Sci. USA **90**:7523–7527.
34. **Kim, J.-J. P., and J. Wu.** 1988. Structure of the medium-chain acyl-CoA dehydrogenase from pig liver mitochondria at 3-Å resolution. Proc. Natl. Acad. Sci. USA **85**:6677–6681.
35. **Komuniecki, R., J. McCrury, J. Thissen, and N. Rubin.** 1989. Electron-transfer flavoprotein from anaerobic *Ascaris suum* mitochondria and its role in NADH-dependent 2-methyl branched-chain enoyl-CoA reduction. Biochim. Biophys. Acta **975**:127–131.
36. **Lee, S. Y., G. N. Bennett, and E. T. Papoutsakis.** 1992. Construction of *Escherichia coli-Clostridium acetobutylicum* shuttle vectors and transformation of *Clostridium acetobutylicum* strains. Biotechnol. Lett. **14**:427–432.
37. **Lee, S. Y., L. D. Mermelstein, G. N. Bennett, and E. T. Papoutsakis.** 1992. Vector construction, transformation, and gene amplification in *Clostridium acetobutylium* ATCC 824. Ann. N. Y. Acad. Sci. **665**:39–51.
38. **Lehman, T. C., D. E. Hale, A. Bhala, and C. Thorpe.** 1990. An acyl-coenzyme A dehydrogenase assay utilizing the ferricenium ion. Anal. Biochem. **186**: 280–284.
39. **Lundberg, N. N., and C. Thorpe.** 1993. Inactivation of short-chain acyl-coenzyme A dehydrogenase from pig liver by 2-pentynoyl-coenzyme A. Arch. Biochem. Biophys. **305**:454–459.
40. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Matsubara, Y., Y. Indo, E. Naito, H. Ozasa, R. Glassberg, J. Vockley, Y. Ikeda, J. Kraus, and K. Tanaka.** 1989. Molecular cloning and nucleotide sequence of cDNAs encoding the precursors of rat long chain acyl-coenzyme A, short chain acyl-coenzyme A, and isovaleryl-coenzyme A dehydrogenases. J. Biol. Chem. **264**:16321–16331.
42. **Matsubara, Y., J. Kraus, H. Ozasa, R. Glassberg, G. Finocchiaro, Y. Ikeda, J. Mole, L. E. Rosenberg, and K. Tanaka.** 1987. Molecular cloning and nucleotide sequence of cDNAs encoding the entire precursors of rat liver medium chain acyl coenzyme A dehydrogenase. J. Biol. Chem. **262**:10104–10108.
43. **Mermelstein, L. D., and E. T. Papoutsakis.** 1993. In vivo methylation in *Escherichia coli* by the *Bacillus subtilis* phage ϕ3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. **59**:1077–1081.
44. **Mermelstein, L. D., N. E. Welker, G. N. Bennett, and E. T. Papoutsakis.** 1992. Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. Bio/Technology **10**:190–195.
45. **Mermelstein, L. D., N. E. Welker, D. J. Peterson, G. N. Bennett, and E. T. Papoutsakis.** 1994. Genetic and metabolic engineering of *Clostridium acetobutylicum* ATCC 824. Ann. N. Y. Acad. Sci. **721**:54–67.
46. **Minami-Ishii, N., S. Taketani, T. Osumi, and T. Hashimoto.** 1989. Molecular cloning and sequence analysis of the cDNA for rat mitochondrial enoyl-CoA hydratase. Eur. J. Biochem. **185**:73–78.
47. **Mullany, P., C. L. Clayton, M. J. Pallen, R. Slone, A. Al-Saleh, and S. Tabaqchali.** 1994. Genes encoding homologues of three consecutive enzymes in the butyrate/butanol-producing pathway of *Clostridium acetobutylicum* are clustered on the *Clostridium difficile* chromosome. FEMS Microbiol. Lett. **124**:61–68.
48. **Naito, E., H. Ozasa, Y. Ikeda, and K. Tanaka.** 1989. Molecular cloning and nucleotide sequence of complementary DNAs encoding human short chain acyl-coenzyme A dehydrogenase and the study of the molecular basis of human short chain acyl-coenzyme A dehydrogenase deficiency. J. Clin. Invest. **83**:1605–1613.
49. **Osumi, T., N. Ishii, M. Hijikata, K. Kamijo, H. Ozasa, S. Furuta, S. Miyazawa, K. Kondo, K. Inoue, H. Kagamiyama, and T. Hashimoto.** 1985. Molecular cloning and nucleotide sequence of the cDNA for rat peroxisomal enoyl-CoA: hydratase-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme. J. Biol. Chem. **260**:8905–8910.
50. **Pace, C. P., and M. T. Stankovich.** 1987. Redox properties of electron-transferring flavoprotein from *Megasphaera elsdenii*. Biochim. Biophys. Acta **911**:267–276.
51. **Powell, P. J., and C. Thorpe.** 1988. 2-Octonoyl coenzyme A is a mechanism-based inhibitor of pig kidney medium-chain coenzyme A dehydrogenase: isolation of the target peptide. Biochemistry **27**:8022–8028.
52. **Pramanik, A., S. Pawar, E. Antonian, and H. Schultz.** 1979. Five different enzymatic activities are associated with the multienzyme complex of fatty acid oxidation from *Escherichia coli*. J. Bacteriol. **137**:469–473.
53. **Raleigh, L. A.** 1984. RbCl transformation procedure for improved efficiency. New England BioLabs Transcript **6**:7.
54. **Rosemann, M. G., A. Liljas, C. I. Branden, and L. J. Banaszak.** 1975. Evolutionary and structural relationship among dehydrogenases, p. 61–102. *In* P. D. Boyer (ed.), The enzymes, vol. 2. Academic Press, Inc., New York.
55. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
56. **Shinzawa, K., T. Inagaki, N. Ohishi, C. Ichihara, N. Tsukagoshi, S. Udaka, and K. Yagi.** 1988. Molecular cloning of a cDNA for α-subunit of rat liver electron transfer flavoprotein. Biochem. Biophys. Res. Commun. **155**:300–304.
57. **Spratt, S. K., P. N. Black, M. M. Ragozzino, and W. D. Nunn.** 1984. Cloning, mapping, and expression of genes involved in the fatty acid degradative multienzyme complex of *Escherichia coli*. J. Bacteriol. **158**:535–542.
58. **Van Berkel, W. J. H., W. A. M. Van Den Berg, and F. Muller.** 1988. Large-scale preparation and reconstitution of apo-flavoproteins with special reference to butyryl-CoA dehydrogenase from *Megasphaera elsdenii*. Eur. J. Biochem. **178**:197–207.
59. **Walter, K. A., G. N. Bennett, and E. T. Papoutsakis.** 1992. Molecular characterization of two *Clostridium acetobutylicum* ATCC 824 butanol dehydrogenase isozyme genes. J. Bacteriol. **174**:7149–7158.
60. **Waterson, R. M., F. J. Castelino, G. M. Hass, and R. L. Hill.** 1972. Purification and characterization of crotonase from *Clostridium acetobutylicum*. J. Biol. Chem. **247**:5266–5271.
61. **Waterson, R. M., and R. L. Hill.** 1972. Enoyl coenzyme A hydratase (crotonase). Catalytic properties of crotonase and its possible regulatory role in fatty acid oxidation. J. Biol. Chem. **247**:5258–5265.
62. **Watmough, N. J., J. Kiss, and F. E. Frerman.** 1992. Structural and redox relationships between *Paracoccus denitrificans*, porcine and human electron-transferring flavoproteins. Eur. J. Biochem. **205**:1089–1097.
63. **Whitfield, C. D., and S. G. Mayhew.** 1974. Purification and properties of electron-transferring flavoprotein from *Peptostreptococcus elsdenii*. J. Biol. Chem. **249**:2801–2810.
64. **Wierenga, R. K., P. Terpstra, and W. G. J. Hol.** 1986. Prediction of the occurrence of the ADP-binding βαβ-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol. **187**:101–107.
65. **Williamson, G., and P. C. Engel.** 1984. Butyryl-CoA dehydrogenase from *Megasphaera elsdenii*: specificity of the catalytic reaction. Biochem. J. **218**: 521–529.
66. **Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks, J. Bonfield, J. Burton, M. Connell, T. Copsey, J. Cooper, A. Coulson, M. Craxton, S. Dear, Z. Du, R. Durbin, A. Favello, A. Fraser, L. Fulton, A. Gardner, P. Green, T. Hawkins, L. Hillier, M. Jier, L. Johnston, M. Jones, J. Kershaw, J. Kirstern, N. Laisster, P. Latreille, J. Lightning, C. Lloyd, B. Mortimore, M. O'Callaghan, J. Parsons, C. Percy, L. Rifken, A. Roopra, D. Saunders, R. Shownkeen, M. Sims, N. Smaldon, A. Smith, M. Smith, E. Sonnhammer, R. Staden, J. Sulston, J. Thierry-Mieg, K. Thomas, M. Vaudin, K. Vaughan, R. Waterson, A. Watson, L. Weinstock, J. Wilkinson-Sproat, and P. Wohldman.** 1994. 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. Nature (London) **368**:32–38.
67. **Yang, S. Y., and M. Elzinga.** 1993. Association of both coenzyme A hydratase and 3-hydroxyacyl coenzyme A epimerase with an active site in the amino-terminal domain of the multifunctional fatty acid oxidation protein from *Escherichia coli*. J. Biol. Chem. **268**:6588–6592.
68. **Yang, X. Y. H., H. Schulz, M. Elzinga, and S.-Y. Yang.** 1991. Nucleotide sequence of the promoter and *fadB* gene of the *fadBA* operon and primary structure of the multifunctional fatty acid oxidation protein from *Escherichia coli*. Biochemistry **30**:6788–6795.
69. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
70. **Youngleson, J. S., D. T. Jones, and D. R. Woods.** 1989. Homology between hydroxybutyryl and hydroxyacyl coenzyme A dehydrogenase enzymes from *Clostridium acetobutylicum* fermentation and vertebrate fatty-acid β-oxidation pathways. J. Bacteriol. **171**:6800–6807.
71. **Youngleson, J. S., F. P. Lin, S. J. Reid, and D. R. Woods.** 1994. Structure and transcription of genes within the β-*hbd-adh1* region of *Clostridium acetobutylicum* P262. FEMS Microbiol. Lett. **125**:185–191.

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Five Different Enzymatic Activities Are Associated with the Multienzyme Complex of Fatty Acid Oxidation from *Escherichia coli*

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The purified multienzyme complex of fatty acid oxidation from *Escherichia coli* was found to possess 3-hydroxyacyl-coenzyme A (CoA) epimerase and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase activities in addition to the previously identified enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoactyl-CoA thiolase activities. Evidence is presented in support of the proposed association of all five enzyme activities with one protein which apparently is composed of two types of subunits and which can exist in several aggregated forms. The five component enzymes of the complex were rapidly inactivated by tris(hydroxymethyl)aminomethane, whereas they remained active in the presence of potassium phosphate.

Fatty acid oxidation in *Escherichia coli* is catalyzed by an inducible enzyme system (13, 17). Acyl coenzyme A (acyl-CoA) synthetase (EC 6.2.1.3), at least two acyl-CoA dehydrogenases (EC 1.3.99.2 and 1.3.99.3), enoyl-CoA hydratase (EC 4.2.1.17), L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), 3-ketoacyl-CoA thiolase (EC 2.3.1.16), 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3), and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase (EC 5.3.3.3) are induced when *E. coli* cells are grown on long-chain fatty acids as the sole carbon source (6, 12, 13, 17). The isolation and mapping of mutants of fatty acid oxidation led to the conclusion that the genes for the enzymes of fatty acid oxidation are located on three separate regions of the *E. coli* chromosome (6). The genes for acyl-CoA synthetase and for the acyl-CoA dehydrogenases were mapped on different locations of the chromosome unlinked to the genes of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA epimerase, and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase, which apparently form an operon (6, 12).

In a previous publication we reported the isolation and purification of a multienzyme complex of fatty acid oxidation from *E. coli* B cells which exhibited enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase activities (2). In the present communication we report that the complex exhibits, additionally, 3-hydroxyacyl-CoA epimerase and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase activities, both of which are required for the degradation of unsaturated fatty acids. We also provide evidence for the association of these five enzyme activities with one protein which can exist in different polymeric forms.

MATERIALS AND METHODS

Materials. The preparation of several substrates and the sources of most materials have been presented recently (2, 3). Polyacrylamide gradient gels were obtained from Pharmacia Fine Chemicals. DL-3-Hydroxydodecanoic acid was synthesized by reduction of ethyl 3-ketododecanoate with $NaBH_4$, followed by alkaline hydrolysis. Ethyl 3-ketododecanoate was prepared by an established procedure (5). *cis*-$\Delta^{3,4}$-Octenoic acid was synthesized from 3-octyn-1-ol obtained from Pfaltz and Bauer by following the procedure of Stoffel and Ecker (16). The CoA derivatives of *cis*-$\Delta^{3,4}$-octenoic acid and DL-3-hydroxydodecanoic acid were prepared by the mixed anhydride method of Goldman and Vagelos (4). Pig heart 3-ketoacyl-CoA thiolase was purified as previously described (15). The conditions of growth of *E. coli* B cells (ATCC 11775) induced for the enzymes of β-oxidation and the purification of the multienzyme complex of fatty acid oxidation have been described previously (2, 3).

Protein and enzyme assays. Protein concentrations were determined by the method of Lowry et al. (8). Thiolase, L-3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase were assayed spectrophotometrically at 303, 340, 263 nm, respectively, as described in principal by Lynen and Ochoa (9) and as detailed previously (3, 14, 17). Acyl-CoA synthetase was assayed by the method of Kornberg and Pricer (7) as described by Overath et al. (12). Acyl-CoA dehydrogenase assays were performed as described by Beinert (1). 3-Hydroxyacyl-CoA epimerase activities were measured spectrophotometrically at 340 nm and at 30°C by an assay in which the epimerase-dependent formation of L-hydroxydodecanoyl-CoA was coupled

to its dehydrogenation and thiolytic cleavage, which were catalyzed by L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, respectively. The assay mixture contained 0.166 M potassium phosphate (pH 8), 0.3 mg of bovine serum albumin per ml, 0.5 mM oxidized nicotinamide adenine dinucleotide, 0.1 mM CoA-sulfhydryl, 60 μM DL-3-hydroxydodecanoyl-CoA, 2 μg of L-3-hydroxyacyl-CoA dehydrogenase per ml, and 1 μg of 3-ketoacyl-CoA thiolase per ml. The reaction was allowed to proceed until the L-isomer of 3-hydroxydodecanoyl-CoA was completely degraded. The epimerase assay was then initiated by the addition of the multienzyme complex of fatty acid oxidation. *cis*-Δ^3-*trans*-Δ^2-Enoyl-CoA epimerase activity was measured spectrophotometrically at 340 nm and at 30°C by an assay in which the isomerase-dependent formation of *trans*-Δ^2-enoyl-CoA was coupled to its hydration, dehydrogenation, and finally thiolytic cleavage, which were catalyzed by crotonase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, respectively. The assay mixture contained 0.166 M potassium phosphate (pH 8), 0.5 mM oxidized nicotinamide adenine dinucleotide, 0.1 mM CoA-sulfhydryl, 30 μM *cis*-Δ^3-octenoyl-CoA, 2.5 μg of 3-ketoacyl-CoA thiolase per ml, 2 μg of L-3-hydroxyacyl-CoA dehydrogenase per ml, and 7.5 μg of crotonase per ml. The reaction was started by the addition of the multienzyme complex of fatty acid oxidation. In homogenates, activities of L-3-hydroxyacyl-CoA dehydrogenase, epimerase, and isomerase were assayed after heating the preparations for 1 min at 70°C to destroy reduced nicotinamide adenine dinucleotide dehydrogenase. One unit of enzyme activity is defined as 1 μmol of substrate converted to product per min.

Gel electrophoresis. Electrophoresis experiments were performed with 4 to 30% polyacrylamide gradient gel slabs (7.6 by 7.5 cm). After application of the protein samples, the gels were subjected to electrophoresis for 5 h at 100 V in a Pharmacia GE-4 electrophoresis apparatus. The electrophoresis buffer was 0.09 M tris(hydroxymethyl)aminomethane (Tris)-boric acid (pH 8.35)–2.5 mM ethylenediaminetetraacetate. After completion of the electrophoresis, either the total slab or narrow slices cut vertically from each side of the slab were stained for 40 min with 1% Coomassie brilliant blue and destained for 1 h in 7% acetic acid, after which the protein bands were visible. In those cases where only two slices of the slab had been stained for protein, the remainder of the gel was cut horizontally into several segments so that each of the main protein bands was associated with a separate slice. All slices were extracted for 18 h in a minimal volume of 0.02 M potassium phosphate (pH 8) containing 10% glycerol, 1 mg of bovine serum albumin per ml, and 5 mM mercaptoethanol.

RESULTS AND DISCUSSION

Enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, three of the seven known enzyme activities required for the degradation of fatty acid to acetyl-CoA in *E. coli*, were found to be associated in a multienzyme complex (2, 10). The purified complex, after heat treatment and chromatography on phosphocellulose, was devoid of acyl-CoA synthetase and acyl-CoA dehydrogenase activities (2). Because the same observation was made when the heat treatment step was omitted, it is concluded that acyl-CoA synthetase and the acyl-CoA dehydrogenases are not component enzymes of the purified multienzyme complex of fatty acid oxidation. However, both 3-hydroxyacyl-CoA epimerase and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase activities were detected in purified preparations of the complex. To further investigate their possible association with the complex, the co-purification of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hydroxyacyl-CoA epimerase, and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase was studied. Results (Table 1) show that all five enzymes were purified to the same extent (36- to 39-fold) when an *E. coli* homogenate was subjected to heat treatment and chromatography on a phosphocellulose column from which not only thiolase, 3-hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase (2), but also epimerase and isomerase, were eluted coincidently. These observations suggest that the epimerase and isomerase are possibly associated with the multienzyme complex of fatty acid oxidation.

When the homogeneity of the purified complex was evaluated by polyacrylamide gradient gel electrophoresis, two intense protein bands

TABLE 1. *Purification of the multienzyme complex of fatty acid oxidation from E. coli*[a]

Enzyme	Substrate	Sp act (μmol/min per mg)		Yield (%)	Purification (-fold)
		Homogenate	Purified protein		
Enoyl-CoA hydratase	Crotonyl-CoA	1.5	57	41	38
L-3-Hydroxyacyl-CoA dehydrogenase	Acetoacetyl-CoA	0.41	16	44	39
3-Ketoacyl-CoA thiolase	Acetoacetyl-CoA	0.05	1.9	42	38
3-Hydroxyacyl-CoA epimerase	D-3-Hydroxydodecanoyl-CoA	0.05	1.8	39	36
cis-Δ^3-*trans*-Δ^2-Enoyl-CoA isomerase	*cis*-Δ^3-Octenoyl-CoA	0.15	5.7	42	38

[a] For experimental details see text.

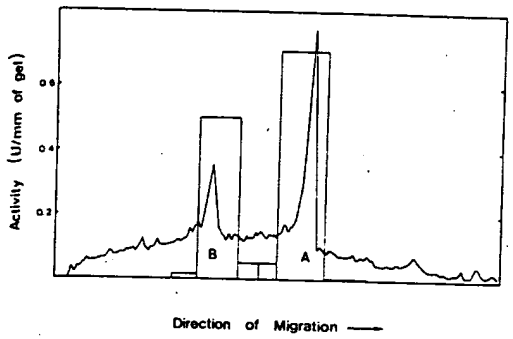

FIG. 1. *Electrophoresis of the purified complex of fatty acid oxidation (0.36 mg) on a 4 to 30% polyacrylamide gradient gel. The recorder tracing was obtained by scanning the absorbance at 500 nm of a gel slice stained for protein. The bars represent enoyl-CoA hydratase activity measured in the extracts of various gel segments. For details see text.*

were observed (Fig. 1). The molecular weights of the two corresponding proteins were estimated by comparison with proteins of known molecular weight to be 265,000 (peak A) and 580,000 (peak B) (Fig. 2). The relative amounts of the two proteins corresponding to peaks A and B differed from one preparation to the next, but the ratio of their molecular weights was consistently found to be approximately 1:2. Because the molecular weight of the multienzyme complex of fatty acid oxidation had been determined previously to be approximately 300,000 (2), peak A must correspond to the complex, whereas peak B was suspected to be due to a dimer of the complex. Preparations of the complex which contained a large amount of the putative dimeric form of the complex were resolved on polyacrylamide gradient gel electrophoresis into three bands (bands A through C), the slowest moving of which (band C) was apparently due to a trimeric form of the complex, with an estimated molecular weight of 820,000 (Fig. 2). Polyacrylamide gel electrophoresis of the purified complex in the presence of sodium dodecyl sulfate demonstrated the presence of only the 42,000- and 78,000-dalton polypeptides previously identified (2). Analysis by two-dimensional gel electrophoresis proved that the proteins corresponding to peaks A and B had identical subunit structures (Fig. 2). Definite proof for the close relationship between proteins A and B (Fig. 1) was obtained when the presence of the same enzymatic activities in these two proteins was established. All five enzymes listed in Table 1 were found to be associated with both protein A and protein B (Fig. 1 and Table 2). The specific and relative activities of the five enzymes observed in regions A and B of the gel are given in Table 2. In view of the low activities present in the gel extracts, the relative activities of the enzymes located in regions A and B of the gel agree reasonably well with those of the starting material (Table 2) except for thiolase, which under a variety of conditions was found to be inactivated more easily than the other enzymes. The activities of all five enzymes in the regions adjacent and between the peaks were either very low or undetectable, as in the case of enzymes with low specific activities. We therefore conclude that epimerase and isomerase, in addition to the three previously identified enzymes of β-oxidation, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, are component enzymes of the multienzyme complex of fatty acid oxidation and that protein B is an enzymatically active dimer of the multienzyme complex of fatty acid oxidation.

During our studies of *E. coli* thiolases we observed and reported (3) that 3-ketoacyl-CoA

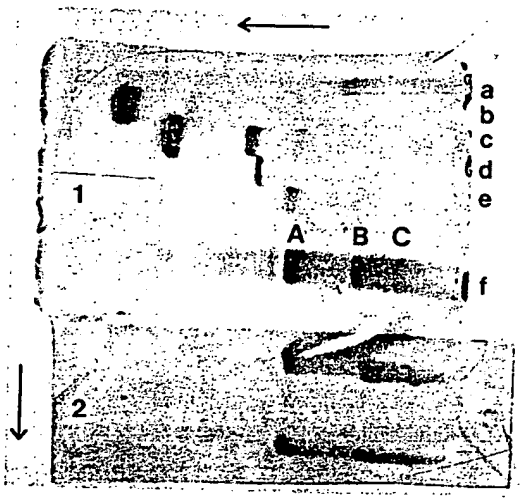

FIG. 2. *Two-dimensional polyacrylamide gel electrophoresis of the multienzyme complex of fatty acid oxidation from E. coli. In gel 1 the native complex and several protein standards were run on a 4% (top) to 30% polyacrylamide gel as described in the text. Before staining the gel, a slice identical to segment f, which contained only the multienzyme complex, was cut off. This slice was incubated with a buffer containing sodium dodecyl sulfate and mercaptoethanol, placed on top of a sodium dodecyl sulfate-polyacrylamide gel (10%, gel 2), and subjected to electrophoresis as described by O'Farrell (11). Arrows indicate directions of migration. Lane a, apoferritin; lane b, ovalbumin; lane c, bovine serum albumin; lane d, lactate dehydrogenase; lane e, catalase; lane f, multienzyme complex of fatty acid oxidation from E. coli. The positions of the monomeric, dimeric, and trimeric forms of the complex are marked as A, B, and C, respectively.*

TABLE 2. *Activities of the component enzymes of the monomer and dimer of the multienzyme complex of fatty acid oxidation from E. coli*[a]

Enzyme	Substrate	Sp act (μmol/min per mm of gel)[d]		Relative activity (%)		
		A[b]	B[b]	PC[c]	A[b]	B[b]
Enoyl-CoA hydratase	Crotonyl-CoA	0.71	0.5	100	100	100
L-3-Hydroxyacyl-CoA dehydrogenase	Acetoacetyl-CoA	0.24	0.21	29	34	42
3-Ketoacyl-CoA thiolase	3-Ketodecanoyl-CoA	0.2	0.15	69	28	30
3-Hydroxyacyl-CoA epimerase	D-3-Hydroxydodecanoyl-CoA	0.085	0.046	10	12	9.1
cis-Δ^3-*trans*-Δ^2-Enoyl-CoA isomerase	*cis*-Δ^4-Octenoyl-CoA	0.021	0.014	3.2	2.9	2.7

[a] Separation of the monomer and dimer of the complex and enzyme assays were performed as described in the text and in the legend to Fig. 1.

[b] A and B, Extracts from regions A and B of polyacrylamide gradient gel (Fig. 1).

[c] PC, Purified complex of fatty acid oxidation. Specific activities of this preparation are given in Table 1 except for thiolase activity with 3-ketodecanoyl-CoA, which was 39.3 U/mg.

[d] Because the gel was divided into unequal slices, the enzyme activities determined in the gel extracts were normalized with respect to the width of the gel slices.

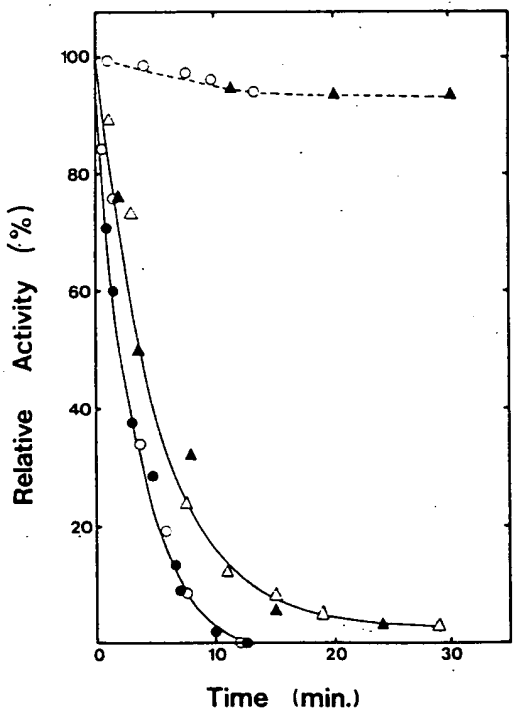

FIG. 3. *Effects of Tris-hydrochloride and potassium phosphate on the activities of the multienzyme complex of fatty acid oxidation. Purified complex (27 μg/ml) at 0°C in the presence of 1 M Tris-hydrochloride pH 8 (——) or 0.2 M potassium phosphate, pH 8 (·····). The activities of the five component enzymes were determined as a function of time. Symbols: ●, thiolase activity with acetoacetyl-CoA; ○, thiolase activity with 3-ketodecanoyl-CoA; ▲, L-3-hydroxyacyl-CoA dehydrogenase with acetoacetyl-CoA; △, L-3-hydroxyacyl-CoA dehydrogenase with 3-ketodecanoyl-CoA. The inactivation curves for enoyl-CoA hydratase with crotonyl-CoA, 3-hydroxyacyl-CoA epimerase, and cis-Δ^3-trans-Δ^2-enoyl-CoA isomerase were virtually identical to that of L-3-hydroxyacyl-CoA dehydrogenase.*

thiolase (thiolase I), which is a component enzyme of the fatty acid oxidation complex (2), was easily inactivated in dilute solution, although it was surprisingly heat stable. Further studies proved that this inactivation was caused by Tris-hydrochloride buffer, which was routinely used for diluting and assaying this enzyme. A systematic study of this phenomenon revealed that all component enzymes of the complex were inactivated by Tris-hydrochloride. The half-times for the inactivations were 2 min for thiolase and 4 to 4.5 min for the other four enzymes when the complex (at a concentration of 27 μg/ml) was kept in 1 M Tris-hydrochloride, pH 8.1 (Fig. 3). However, in 0.2 M potassium phosphate (pH 8) none of the enzymes was significantly inactivated (Fig. 3). The inactivation was slower and not complete when the concentration of the protein was higher or when that of Tris-hydrochloride was lower. Because the Tris-dependent inactivation of long-chain enoyl-CoA hydratase was slower than that of crotonase and because the ratio of short-chain to long-chain enoyl-CoA hydratase activities did not remain constant during the purification of the protein, the possibility exists that two enoyl-CoA hydratases are present in the complex. There is no evidence for the presence of more than one thiolase or one L-3-hydroxyacyl-CoA dehydrogenase in the complex.

We conclude that in *E. coli* the five enzymes enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, 3-hy-

droxyacyl-CoA epimerase, and *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase, whose genes are closely linked and possibly form an operon (12), are associated in a multienzyme complex of fatty acid oxidation. Further studies should establish the quaternary structure of the complex as well as the physiological significance of the arrangement of several, but not all, enzymes of β-oxidation in a multienzyme complex.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Beinert, H.** 1962. Acyl dehydrogenases from pig and beef liver and beef heart. Methods Enzymol. 5:546–557.
2. **Binstock, J. F., A. Pramanik, and H. Schulz.** 1977. Isolation of a multi-enzyme complex of fatty acid oxidation from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 74:492–495.
3. **Feigenbaum, J., and H. Schulz.** 1975. Thiolases of *Escherichia coli*: purification and chain length specificities. J. Bacteriol. 122:407–411.
4. **Goldman, P., and P. R. Vagelos.** 1961. The specificity of triglyceride synthesis from diglycerides in chicken adipose tissue. J. Biol. Chem. 236:2620–2623.
5. **Hunsdiecker, H.** 1942. Über das Verhalten der γ-Diketone. Chem. Ber. 75:447–468.
6. **Klein, K., R. Steinberg, B. Fiethen, and P. Overath.** 1971. Fatty acid degradation in *Escherichia coli*. An inducible system for the uptake of fatty acids and further characterization of *old* mutants. Eur. J. Biochem. 19:442–450.
7. **Kornberg, A., and W. E. Pricer.** 1953. Enzymatic esterification of α-glycerophosphate by long chain fatty acids. J. Biol. Chem. 204:345–357.
8. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
9. **Lynen, F., and S. Ochoa.** 1953. Enzymes of fatty acid metabolism. Biochim. Biophys. Acta 12:299–314.
10. **O'Brien, W. J., and F. E. Frerman.** 1977. Evidence for a complex of three beta-oxidation enzymes in *Escherichia coli*: induction and localization. J. Bacteriol. 132: 532–540.
11. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007–4021.
12. **Overath, P., G. Pauli, and H. U. Schairer.** 1969. Fatty acid degradation in *Escherichia coli*. An inducible acyl-CoA synthetase, the mapping of *old*-mutations and the isolation of regulatory mutants. Eur. J. Biochem. 7: 559–574.
13. **Overath, P., E. M. Raufuss, W. Stoffel, and W. Ecker.** 1967. The induction of the enzymes of fatty acid degradation in *Escherichia coli*. Biochem. Biophys. Res. Commun. 29:28–33.
14. **Schulz, H.** 1974. Long chain enoyl coenzyme A hydratase from pig heart. J. Biol. Chem. 249:2704–2709.
15. **Staack, H., J. F. Binstock, and H. Schulz.** 1978. Purification and properties of a pig heart thiolase with broad chain length specificity and comparison of thiolases from pig heart and *Escherichia coli*. J. Biol. Chem. 253:1827–1831.
16. **Stoffel, W., and W. Ecker.** 1969. Δ^3-*cis*, Δ^2-*trans*-enoyl-CoA isomerase from rat liver mitochondria. Methods Enzymol. 14:99–109.
17. **Weeks, G., M. Shapiro, R. O. Burns, and S. J. Wakil.** 1969. Control of fatty acid metabolism. I. Induction of the enzymes of fatty acid oxidation in *Escherichia coli*. J. Bacteriol. 97:827–836.

> *Fukui & Doi, "Cloning and analysis of the
> poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of
> Aeromonas caviae," J Bacteriol. 179(15):4821-30 (1997). AC

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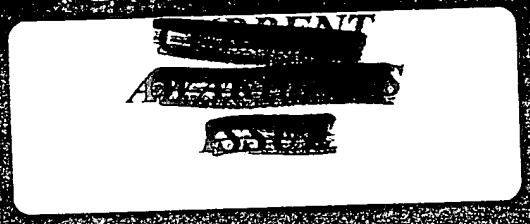

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Cloning and Analysis of the Poly(3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) Biosynthesis Genes of *Aeromonas caviae*

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A 5.0-kbp *Eco*RV-*Eco*RI restriction fragment was cloned and analyzed from genomic DNA of *Aeromonas caviae*, a bacterium producing a copolyester of (*R*)-3-hydroxybutyrate (3HB) and (*R*)-3-hydroxyhexanoate (3HHx) [P(3HB-*co*-3HHx)] from alkanoic acids or oils. The nucleotide sequence of this region showed a 1,782-bp poly (3-hydroxyalkanoate) (PHA) synthase gene ($phaC_{Ac}$ [i.e., the *phaC* gene from *A. caviae*]) together with four open reading frames (ORF1, -3, -4, and -5) and one putative promoter region. The cloned fragments could not only complement PHA-negative mutants of *Alcaligenes eutrophus* and *Pseudomonas putida*, but also confer the ability to synthesize P(3HB-*co*-3HHx) from octanoate or hexanoate on the mutants' hosts. Furthermore, coexpression of ORF1 and ORF3 genes with $phaC_{Ac}$ in the *A. eutrophus* mutant resulted in a decrease in the polyester content of the cells. *Escherichia coli* expressing ORF3 showed (*R*)-enoyl-coenzyme A (CoA) hydratase activity, suggesting that (*R*)-3-hydroxyacyl-CoA monomer units are supplied via the (*R*)-specific hydration of enoyl-CoA in *A. caviae*. The transconjugant of the *A. eutrophus* mutant expressing only $phaC_{Ac}$ effectively accumulated P(3HB-*co*-3HHx) up to 96 wt% of the cellular dry weight from octanoate in one-step cultivation.

The utilization of biological systems for production of biodegradable materials is becoming important as a solution of the problems concerning plastic waste and the global environment. Poly(3-hydroxyalkanoates) (PHA) are produced by a wide variety of bacteria as intracellular carbon- and energy-storage materials from renewable carbon resources, such as sugars or plant oils (2, 6, 18, 25). Since these bacterial PHA are biodegradable thermoplastics, they have attracted industrial attention as possible candidates for large-scale biotechnological products. At present, more than 90 different monomeric units have been found as constituents of PHA (37).

Bacterial PHA can be divided into two groups, depending on the number of carbon atoms in the monomeric units (35). One group of bacteria, including *Alcaligenes eutrophus*, produces short-chain-length PHA with C_3-to-C_5 monomer units, while the other group, including *Pseudomonas oleovorans*, synthesizes medium-chain-length PHA with C_6-to-C_{14} monomer units. Only a few reports are available for bacteria which can synthesize PHA consisting of both short- and medium-chain-length monomer units. For example, *Rhodospirillum rubrum* (3), *Rhodocyclus gelatinosus* (19), and *Rhodococcus ruber* (12) produce terpolymers consisting of C_4, C_5, and C_6 3-hydroxyalkanoate (3HA) units from hexanoate, and some pseudomonad strains accumulate PHA consisting of C_4-to-C_{12} 3HA units (16, 38). Our laboratory has found that a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx), P(3HB-*co*-3HHx), is produced by *Aeromonas caviae* FA440 isolated from soil (7, 32). This bacterium synthesizes the copolyester from alkanoic acids of even carbon numbers or from plant oils up to approximately 30 wt% of the cellular dry weight, with a 3HHx fraction ranging from 10 to 25 mol%. We have also demonstrated that P(3HB-*co*-3HHx) is a flexible material and that films of the copolymer show a high degree of elongation to break (<850%) (7).

PHA biosynthesis genes, including structural genes of PHA synthases, have been isolated and analyzed at a molecular level from various sources (18, 36). The genes of *A. eutrophus* are organized in a single operon as *phbC-A-B*, which are genes of PHA synthase, β-ketothiolase, and NADPH-acetoacetyl-coenzyme A (CoA) reductase, respectively (26, 31, 34). Whereas in *P. oleovorans*, two structural genes of PHA synthases (*phaC1* and *phaC2*) flanking a PHA depolymerase gene have been identified (14), PHA synthases of *Chromatium vinosum* (21) and *Thiocapsa pfennigii* (35) consist of two different types of subunits encoded by *phbC* and *phbE* in a single operon. Although cells of *T. pfennigii* accumulated only P(3HB) homopolymer from various carbon sources, a recombinant *Pseudomonas putida* strain harboring the PHA biosynthesis genes of *T. pfennigii* produced a new type of PHA consisting of 3HB, 3HHx, and 3-hydroxyoctanoate units from octanoate (20) or of PHA containing 4-hydroxy- and 5-hydroxyalkanoate units from the related carbon sources (40, 41). In this study, we cloned and sequenced the P(3HB-*co*-3HHx) biosynthesis genes of *A. caviae* FA440 to study the molecular organization. In addition, heterologous expression of the cloned genes was examined in PHA-negative mutants of *A. eutrophus* and *P. putida* to characterize the genes and the PHA-producing ability of the recombinant strains was investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *A. caviae*, *A. eutrophus*, and *P. putida* strains were cultivated at 30°C in a nutrient-rich medium containing 10 g of meat extract, 10 g of polypeptone, and 2 g of yeast extract in 1 liter of distilled water, and *Escherichia coli* strains were grown at 37°C on a Luria-Bertani medium (28). When needed, kanamycin (50 mg/liter) or ampicillin (50 mg/liter) was added to maintain the plasmids.

DNA manipulation. Isolation of total genomic DNA and plasmids, digestion of DNA with restriction endonucleases, and transformation of *E. coli* were carried out by standard procedures (28). Transconjugation of *A. eutrophus* or *P. putida*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
A. caviae FA440	Wild type	FERM P-3432
A. eutrophus PHB$^-$4	PHA-negative mutant of H16	7, 32
P. putida GPp104	PHA-negative mutant of KT2442	DSM 541, 30
E. coli		14
DH5α	*deoR endA1 gyrA96 hsdR17* (r_K^- m_K^+) *recA1 relA1 supE44 thi-1* Δ(*lacZYA-argFV169*) ϕ80Δ*lacZ*ΔM15 F^- λ^-	Clontech
S17-1	*recA* and *tra* genes of plasmid RP4 integrated into the chromosome; auxotrophic for proline and thiamine	33
Plasmids		
pLA2917	Cosmid; Km^r Tc^r RK2 replicon Mob$^+$	1
pJRD215	Cosmid; Km^r Sm^r RSF1010 replicon Mob$^+$	4
pJRDEE50	pJRD215 derivative; $phaC_{Ac}$ ORF1 ORF3 ORF4 ORF5	This study
pJRDEE32	pJRD215 derivative; $phaC_{Ac}$ ORF1 ORF3	This study
pBluescriptII KS$^+$	Ap^r *lacPOZ* T7 and T3 promoter	Stratagene
pUC18	Ap^r *lacPOZ*	Takara
pEE32	pUC18 derivative; $phaC_{Ac}$ ORF1 ORF3	This study

with *E. coli* S17-1 harboring broad-host-range plasmids was performed as described by Friedrich et al. (8).

PCR. To amplify a partial fragment of the *A. caviae* PHA synthase gene from genomic DNA, we performed PCR with two primers [P1, 5'-CC(C/G)CC(C/G)TGGATCAA(T/C)AAGT(T/A)(T/C)TA(T/C)ATC-3'; P2, 5'-(G/C)AGCCA(G/C)GC(G/C)GTCCA(A/G)TC(G/C)GGCCACCA-3'] under 25 cycles of denaturation at 98°C for 20 s and annealing and elongation at 65°C for 2 min. The amplified DNA was purified by phenol extraction and ethanol precipitation.

Cloning of genes. Genomic DNA was partially digested with *Sau*3AI and ligated to the cosmid vector pLA2917 (1) linearized with *Bgl*II. The concatemeric ligation products were packaged by using Gigapack II (Stratagene), and the resultant phage particles were transfected to *E. coli* S17-1. The constructed cosmid library was screened by colony hybridization (28) with the PCR-amplified fragment as a probe to isolate the PHA biosynthesis genes of *A. caviae*. Preparation of the labeled probe and detection of the hybridization signals on membranes were carried out with a digoxigenin nucleic acid labeling and detection kit (Boehringer Mannheim).

DNA sequencing analysis. DNA fragments were subcloned into pBluescriptII KS$^+$ or pUC18, and nested sets of deletion clones were generated by using exonuclease III for DNA sequencing (28). DNA was sequenced in a DSQ-1000 DNA sequencer (Shimadzu Co., Kyoto, Japan) with a *Taq* cycle sequencing kit (Takara Co., Kyoto, Japan). Computer analysis of the resulting nucleotide sequence was performed with SDC-GENETYX genetic information processing software (Software Development Co., Tokyo, Japan).

Site-directed mutagenesis. To create restriction sites in the isolated genes, site-directed mutagenesis was carried out under the unique site elimination procedure developed by Deng and Nickoloff (5) with a U.S.E. mutagenesis kit (Pharmacia). Primers M1 and M2 (used for creation of *Bgl*II sites) and primers M3 and M4 (used for creation of *Bam*HI sites) were as follows: M1, 5'-GCCGATTGCCCCAGATCTACACTGTTCTGCC-3'; M2, 5'-GACGCTACGGGCTAGATCTCGCCTCGGGTGTG-3'; M3, 5'-CGCATGAGCGCAGGATCCCTGGAAGTAGGC-3'; and M4, 5'-GCCGTGACGGGGGGATCCGTGGTCAAGCTG-3'.

Production and analysis of PHA. One-step production of polyesters was carried out on a reciprocal shaker (130 strokes/min) at 30°C for 72 h in 500-ml flasks with 100 ml of a nitrogen-limited mineral salt medium, which was composed of 0.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.15 g of KH_2PO_4, 0.05 g of NH_4Cl, 0.02 g of $MgSO_4 \cdot 7H_2O$, and 0.1 ml of trace element solution (16). In the case of two-step production, cells were first cultivated in 100 ml of nutrient-rich medium for 12 h. Harvested and washed cells were then transferred into a nitrogen (NH_4Cl)-free mineral salt medium and incubated at 30°C for 48 h. Filter-sterilized carbon sources were added as indicated in the text. For maintenance of broad-host-range plasmids in *A. eutrophus* or *P. putida*, kanamycin was added to the medium at a concentration of 50 mg/liter. Cellular PHA content and composition were determined by gas chromatography after methanolysis of dried cells in the presence of 15% sulfuric acid, as described previously (16).

PHA synthase assay. Crude cell extracts of *A. eutrophus* transconjugants were prepared as described by Schubert et al. (31). The activity of PHA synthase was determined by spectroscopic assay according to the methods described by Valentin and Steinbüchel (43). (*R*)-3HB-CoA was synthesized by the mixed anhydride method described by Haywood et al. (11).

Enoyl-CoA hydratase assay. Recombinant *E. coli* cells were sonicated and centrifuged (20,000 × *g*, 20 min, 4°C), and the resulting soluble cell extracts were used as an enzyme solution. Enoyl-CoA hydratase activity was assayed by the hydration of crotonyl-CoA (Sigma) followed by measurement of the disappearance of absorbance at 263 nm derived from the decrease of the enoyl-thioester bond, as described by Moskowitz and Merrick (23). The configuration of 3HB-CoA produced by the hydratase was determined by coupling with the (*S*)-specific dehydrogenation. NAD^- and (*S*)-3HA-CoA dehydrogenase (Sigma) were added after the hydration reaction had reached equilibrium, and the reduction of NAD^- was monitored at 340 nm (23).

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. D88825.

RESULTS

Identification and cloning of the *A. caviae* PHA biosynthesis genes. For the identification of the PHA biosynthesis genes of *A. caviae*, a partial fragment of a PHA synthase gene was amplified from the genomic DNA and used as a specific probe. Two primers were designed from highly conserved regions among known PHA synthases (244-PPWINK(Y/F)YI-252 and 547-WWPDWTAWL-555; numbering corresponds to the *A. eutrophus* PHA synthase) (36), and PCR with the designed primers resulted in successful amplification of an approximately 900-bp fragment. Hybridization analysis gave only one positive signal for each of the *Eco*RI-, *Bam*HI-, *Hin*dIII-, and *Pst*I-digested genomic DNAs of *A. caviae* with a probe prepared from the amplified fragment. There were no detectable hybridization signals even under low-stringency conditions when the PHA synthase gene of *A. eutrophus* (26, 31, 34) was used as a probe. A cosmid library constructed in *E. coli* S17-1 was screened by colony hybridization, and one positive recombinant clone was isolated, which harbored a 20-kbp *A. caviae* genomic DNA fragment. A positive 11-kbp *Sal*I subfragment (SS110) was cloned into pBluescriptII KS$^+$, and further analysis showed that the PHA biosynthesis genes of *A. caviae* were located in a 5.0-kbp *Eco*RV-*Eco*RI subfragment, referred to as VE50.

Nucleotide sequence and structure of the PHA biosynthesis genes. A nucleotide sequence of the VE50 fragment was determined for both strands. Fig. 1b and 2 show the restriction map and the determined nucleotide sequence, respectively, of the VE50 fragment. Five open reading frames (ORFs) were identified (ORF1 to -5) in the fragment by computer analysis, as shown in Fig. 1c.

ORF2 (1,782 bp), which is the largest gene in this fragment, encoded a protein composed of 594 amino acids with a mo-

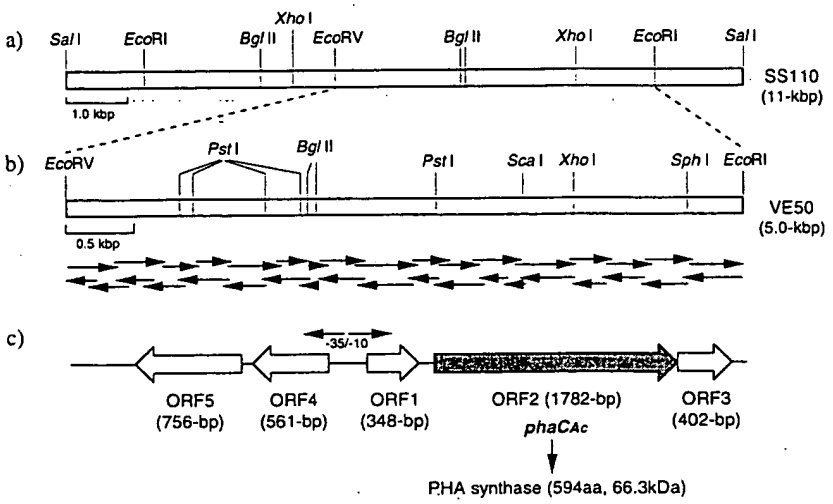

FIG. 1. Organization of *A. caviae* PHA biosynthesis genes, restriction endonuclease sites, and DNA sequencing strategy. (a) Restriction map of SS110 fragment. (b) Restriction map and sequencing strategy of VE50 subfragment. Arrows indicate sequence strategy. (c) Organization of $phaC_{Ac}$. ORF1, ORF3, ORF4, and ORF5. aa, amino acids.

lecular mass of 66,334 Da. Figure 3 shows a partial alignment and the identities of its deduced amino acid sequence with known PHA synthases from 11 microorganisms. Relatively high identities were obtained with the synthases of *Acinetobacter* sp. (45.1%) (29) and *A. eutrophus* (42.7%); therefore, ORF2 was concluded to represent a structural gene of *A. caviae* PHA synthase, and it was referred to as $phaC_{Ac}$. The calculated molecular mass of the translated PHA synthase was almost consistent with that of *A. eutrophus* (63,940 Da) and those of *P. oleovorans* (62,400 and 62,600 Da) (14). The propagation of a polyester chain has been proposed to include the formation of an acyl-*S* enzyme intermediate at two thiol groups and the transesterification to a propagating chain (17). Cys-319 in the *A. eutrophus* synthase has been demonstrated by mutagenic analysis to play important roles in the catalytic cycles (9), and the corresponding Cys residue is conserved in the PHA synthase of *A. caviae* at the same position, 319, in a lipase box-like sequence. Another active site has been proposed to be a thiol group of a 4′-phosphopantetheine moiety which post-translationally modifies the synthase of *A. eutrophus* in *E. coli* (9). A candidate for the modified residue, Ser-260, is also found in the amino acid sequence deduced from $phaC_{Ac}$.

ORF1 (348 bp) and ORF3 (402 bp) were located in upstream and downstream regions, respectively, of $phaC_{Ac}$, and ORF4 (561 bp) and ORF5 (756 bp) were oriented in the opposite direction to the other three genes. Several −35 to −10 consensus sequences of a σ^{70}-dependent promoter (10) were found between ORF1 and ORF4 on both strands, suggesting that the approximately 300-bp region is a putative promoter region for these five genes. The ATG start codon of ORF3 overlapped with the TGA stop codon of $phaC_{Ac}$, and an inverted repeat, which may serve as a transcriptional termination signal, was identified in the downstream region of ORF3 (nucleotides 4899 to 4930) with the structural free energy of −166 kJ/mol. The presence of ribosome binding sequences 5 to 8 bp upstream of the ATG start codon of all genes suggests the translation of these five genes.

Complementation studies and heterologous expression. To confirm whether the cloned fragments harbor functionally active PHA biosynthesis genes, heterologous expression of the genes was investigated in the PHA-negative mutants PHB$^-$4 of *A. eutrophus* (30) and GPp104 of *P. putida* (14). The VE50 fragment (containing $phaC_{Ac}$, ORF1, ORF3, ORF4, and ORF5) and a 3.2-kbp *Bgl*II-*Eco*RI fragment (containing $phaC_{Ac}$, ORF1, and ORF3), both of which harbored the putative promoter region, were converted to *Eco*RI restriction fragments referred to as EE50 and EE32, respectively, with a p*Eco*RI linker. These two fragments were inserted into a broad-host-range vector, pJRD215 (4), at the unique *Eco*RI site, and the resultant plasmids, pJRDEE50 and pJRDEE32, were mobilized from *E. coli* S17-1 to *A. eutrophus* PHB$^-$4 or to *P. putida* GPp104. The transconjugants were cultivated in a mineral salt medium to promote the PHA biosynthesis from sugar (fructose for transconjugants of PHB$^-$4 or gluconate for those of GPp104), hexanoate, or octanoate as a carbon source.

Table 2 shows the results of PHA accumulation in the recombinant strains by one-step cultivation. The plasmids pJRDEE50 and pJRDEE32 not only could complement the deficiency of PHA synthase in the mutant strains but also could confer the ability to synthesize P(3HB-*co*-3HHx) copolymer on the hosts. *A. eutrophus* PHB$^-$4 harboring pJRDEE32 produced P(3HB) homopolymer from fructose (53 wt% of the cellular dry weight) and P(3HB-*co*-3HHx) copolymer with 22 mol% of the 3HHx fraction from octanoate (33 wt%), while the strain harboring pJRDEE50 accumulated only a small amount of polyesters (0 to 7 wt%). *P. putida* GPp104 harboring these plasmids accumulated more than 40 wt% of P(3HB-*co*-3HHx) from hexanoate or octanoate, with a high 3HHx composition. The mole fraction of the 3HHx unit reached 40 mol% in the hexanoate-grown cells. These strains synthesized P(3HB-*co*-3HHx) not only from the carboxylic acids but also from gluconate, although the content was low (4 wt%).

Furthermore, ORF1 and/or ORF3 were deleted from the EE32 fragment, and the accumulation of PHA in transconjugants of PHB$^-$4 harboring the deleted clones was investigated. The EE32 fragment was ligated to pUC18 to form a recombinant plasmid called pEE32. Two *Bgl*II sites across the coding region of ORF1 or two *Bam*HI sites across that of ORF3 were created by site-directed mutagenesis, and elimination of the *Bgl*II or *Bam*HI fragment from the modified plasmids gave pEE32d1 and pEE32d3, respectively. pEE32d13 was also constructed by eliminating both the *Bgl*II and *Bam*HI fragments. The deleted *Eco*RI restriction fragments of these plasmids were inserted into pJRD215, resulting in the formation of

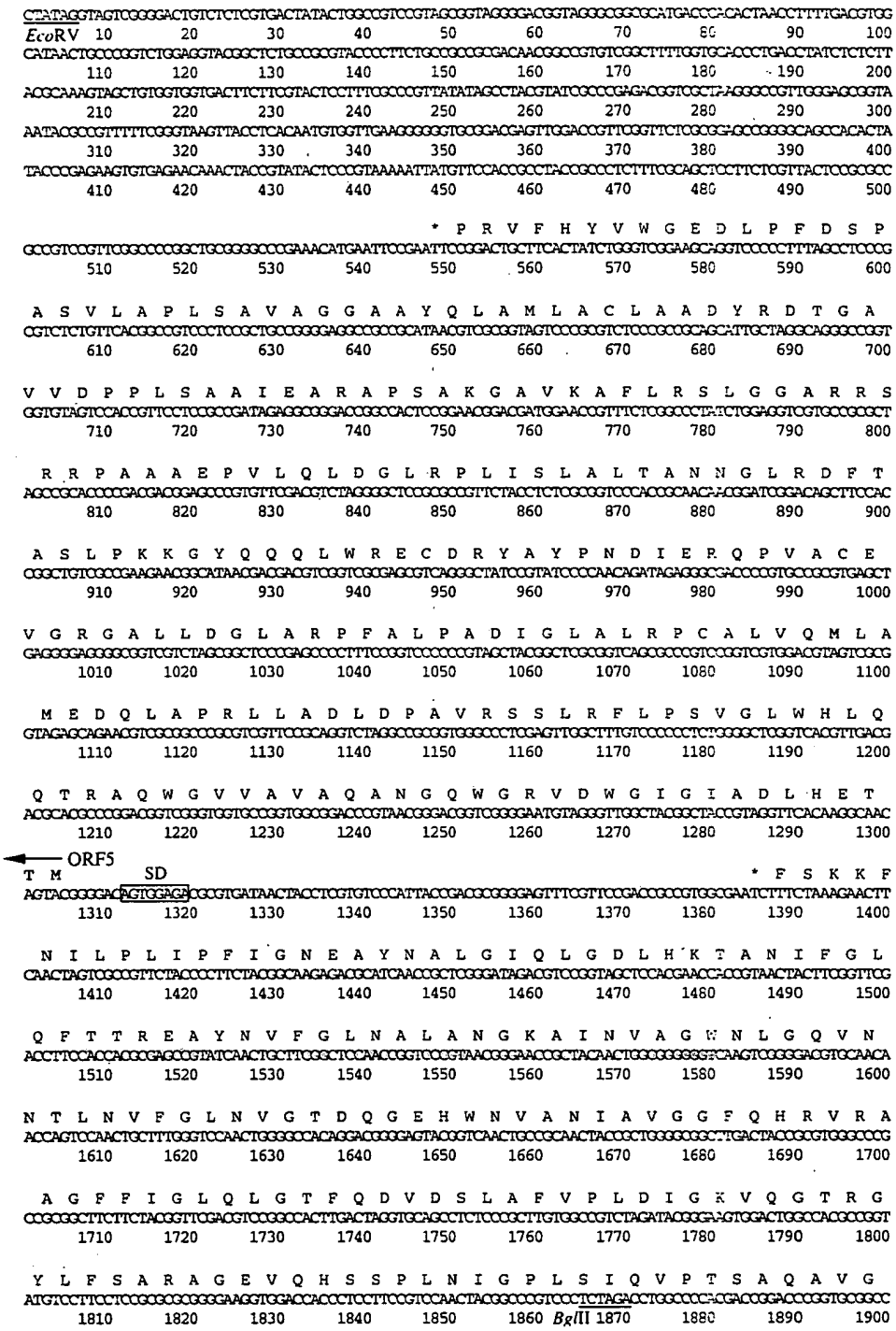

FIG. 2. Nucleotide sequence of a 5.051-bp region containing the $phaC_{Ac}$ gene along with the deduced amino acid sequence. The putative ribosome binding sequences are boxed and indicated by SD. The −10 and −35 regions of the σ^{70}-dependent promoter are labeled. A potential stem-loop structure is shown by the facing arrows. P1 and P2 indicate the positions of the primers used for PCR. M1, M2, M3, and M4 indicate the restriction sites created by site-directed mutagenesis. Other restriction sites are underlined and labeled. Stop codons are indicated by asterisks.

pJRDEE32d1, harboring $phaC_{Ac}$ and ORF3, pJRDEE32d3, harboring $phaC_{Ac}$ and ORF1, and pJRDEE32d13, harboring only $phaC_{Ac}$. *A. eutrophus* PHB$^-$4 was transformed by conjugational transfer of these recombinant plasmids, and the transconjugants were cultivated in a nitrogen-limited mineral salt medium. As shown in Table 3, all the transconjugants of PHB$^-$4 accumulated P(3HB) homopolymer from fructose, and the deletion of ORF1 and/or ORF3 slightly increased the PHA content in the cells. When the recombinant PHB$^-$4 strains were grown on octanoate or hexanoate, the content of P(3HB-*co*-3HHx) copolymer was remarkably increased by the deletion of ORFs. Especially on octanoate, the PHA content

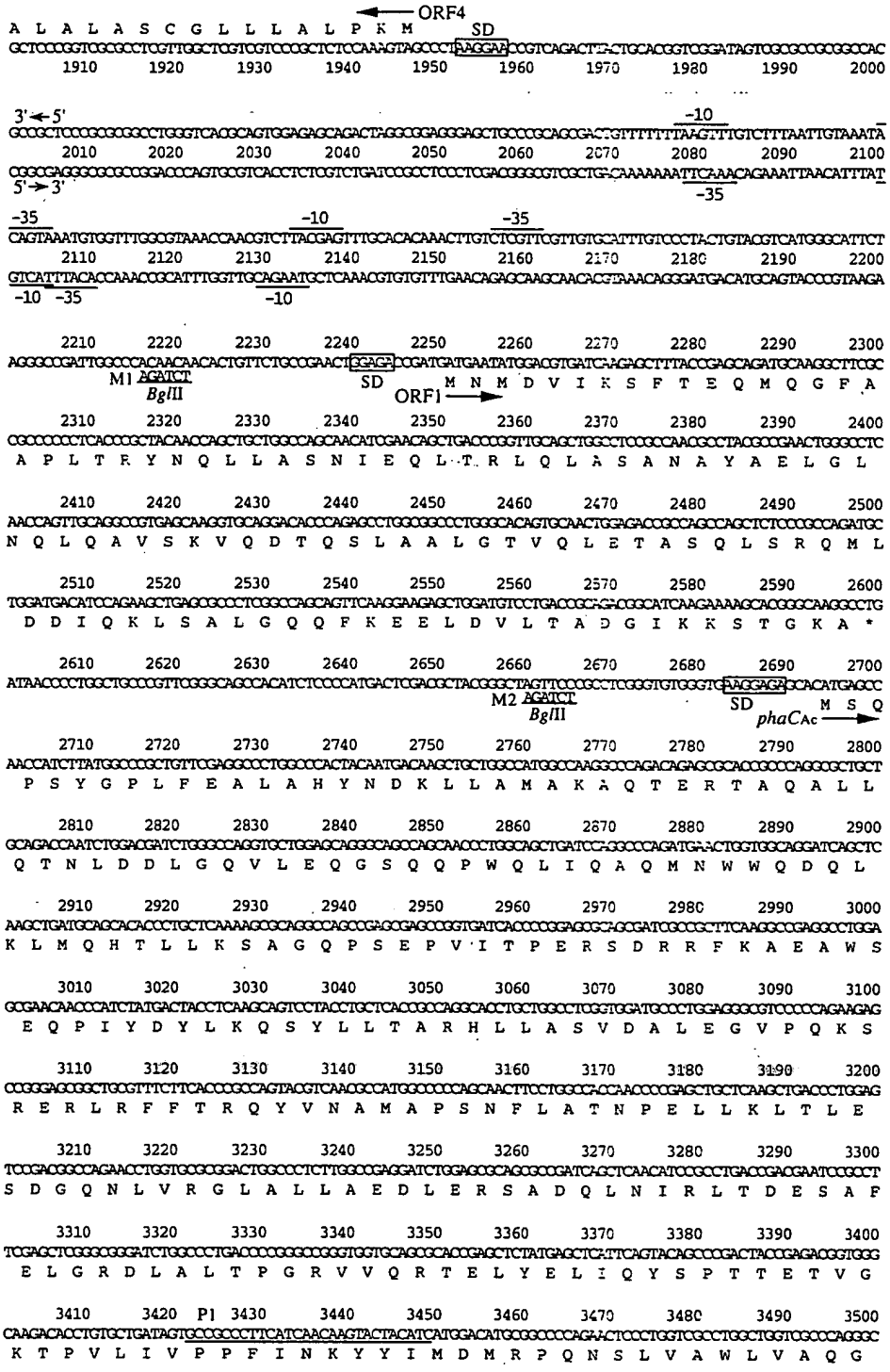

FIG. 2—*Continued.*

reached 92% of the cell weight (dry wt) after deletion of ORF1 or ORF3 from the EE32 fragment, and 96% (dry wt) after deletion of both genes. A similar tendency was also observed in the case of two-step cultivation.

Table 4 shows PHA synthase activities in the transconjugants of PHB$^-$4 at the early stationary growth phase. When octanoate was fed as a sole carbon source. the PHA synthase activity in the cells expressing $phaC_{Ac}$ with ORF1 (pJRDEE32d3) was fivefold higher than that in the cells coexpressing the three genes (pJRDEE32). Furthermore, the strain expressing only $phaC_{Ac}$ without both ORF1 and ORF3 (pJRDEE32d13) showed 38-fold higher activity than that harboring pJRDEE32. In contrast, the transconjugant harboring pJRDEE32d1 showed lower activities in spite of the high PHA content than did those harboring pJRDEE32 or pJRDEE32d3. The three strains harboring these deleted plasmids accumulated P(3HB-*co*-3HHx)

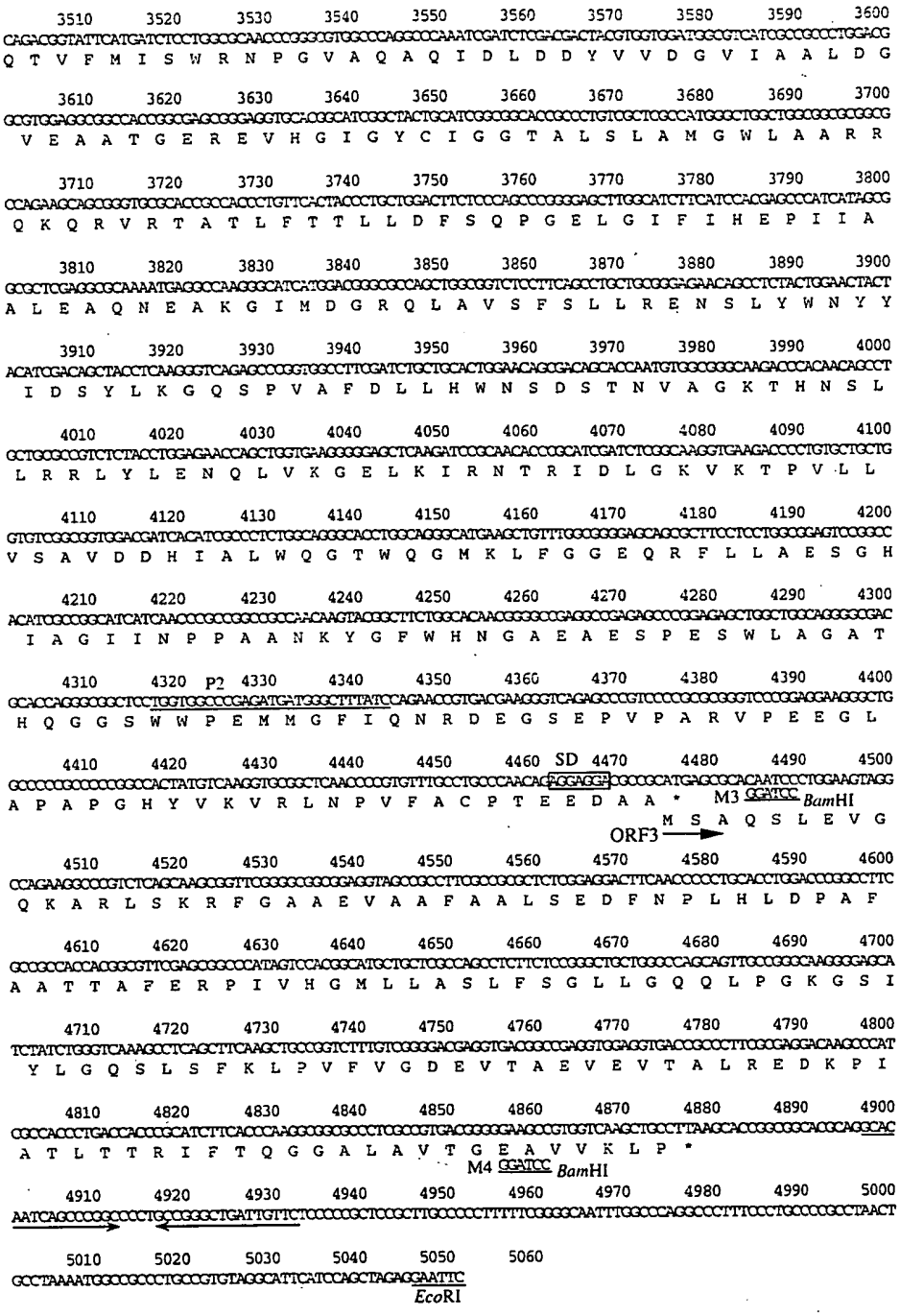

FIG. 2—*Continued.*

up to more than 92 wt% from octanoate after 72 h cultivation, but they showed quite different synthase activities. These results suggested that the PHA synthase activity does not relate directly to the PHA content accumulated in cells.

The deletion of ORFs from the EE32 fragment did not seriously affect the composition of copolyesters accumulated in octanoate-grown cells (ranging from 12 to 22 mol% of the 3HHx fraction). However, it is of interest to note that the mole fractions of the 3HHx unit in copolyesters synthesized from hexanoate were increased by the deletion of ORF3. P(3HB-*co*-3HHx), consisting of almost equimolar amounts of 3HB and 3HHx units, could be obtained by two-step cultivation of the strains harboring pJRDEE32d3 or pJRDEE32d13.

Enoyl-CoA hydratase assay. No significant identities of the amino acid sequences deduced from the nucleotide sequences of identified ORFs were detected with those of primary structures of any proteins in databases, except for the translated product of ORF3, which showed a partial identity with a putative enoyl-CoA hydratase domain of *Saccharomyces cerevisiae* β-oxidation multifunctional protein (38.4% of 73 amino acids) (13). The ORF3 product also exhibited a weak identity with *Clostridium difficile* crotonase (22.8% of 114 amino acids)

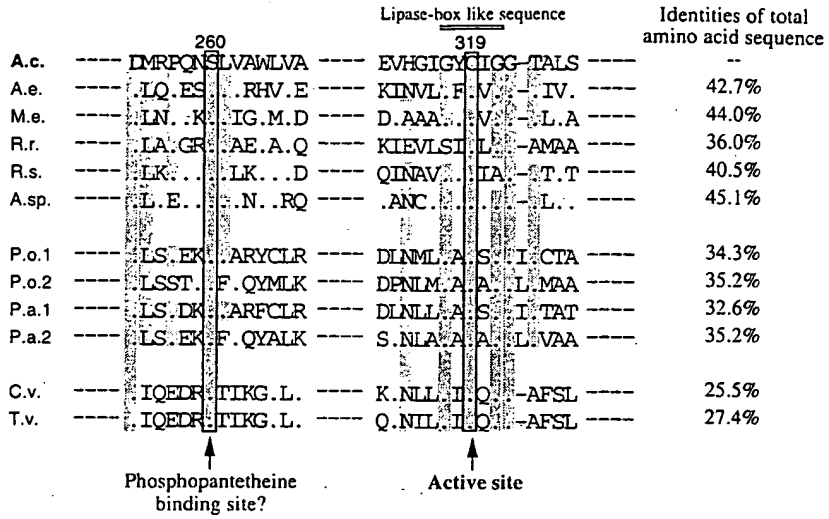

FIG. 3. Partial alignment and identities of the deduced amino acid sequence of the PHA synthase from *A. caviae* with those from *A. eutrophus* (A.e.) (26, 31, 34), *Methylobacterium extorquens* (M.e.) (42), *R. ruber* (R.r.) (27), *R. sphaeroides* (R.s.) (15), *Acinetobacter* sp. (A.sp.) (29), *P. oleovorans* (P.o.1 and P.o.2) (14), *P. aeruginosa* (P.a.1 and P.a.2) (39), *C. vinosum* (C.v.) (21), and *Thiocystis violacea* (T.v.) (22). Dots, amino acids identical to the *A. caviae* sequence; shading, amino acids which are identical in at least eight PHA synthases.

(24). Therefore, soluble extracts of recombinant *E. coli* harboring the PHA biosynthetic genes of *A. caviae* were prepared, and enoyl-CoA hydratase activity was assayed with crotonyl-CoA as a substrate. The results are given in Table 5. *E. coli* harboring pEE32 or pEE32d1 exhibited a high enoyl-CoA hydratase activity, while the activity in the cells harboring pEE32d3 or pEE32d13 was as low as that in a control strain harboring pUC18. To investigate the stereospecificity of the hydration reaction, NAD^+ and (*S*)-3HA-CoA dehydrogenase were added to the reaction mixture after the hydration of crotonyl-CoA had reached equilibrium (23), but the formation of NADH linked with the oxidation of (*S*)-3HB-CoA was not observed. These results strongly suggest that ORF3 is a structural gene of (*R*)-specific enoyl-CoA hydratase. The β-oxidation multifunctional protein of *S. cerevisiae* encoded by the *fox2* gene has been reported to catalyze the hydration of enoyl-CoA with (*R*)-specificity (13). The evolutionary relationship between *A. caviae* ORF3 and *S. cerevisiae fox2* may be an interesting subject.

DISCUSSION

There are only a few studies on bacteria capable of incorporating both short- and medium-chain-length 3HA units into polyester chains. *R. ruber* (12) and *R. rubrum* (3) are known to synthesize PHA with C_4-to-C_6 3HA units, and the PHA synthase genes of these bacteria have been cloned (15, 27). However, the substrate specificities of the translated PHA synthases were not investigated thoroughly. In this study, cloning of PHA biosynthesis genes, including a structural gene of PHA synthase from *A. caviae* ($phaC_{Ac}$), was performed together with an investigation of the PHA-producing ability of recombinant strains harboring the cloned genes.

A partial fragment of a PHA synthase gene was successfully amplified from genomic DNA of *A. caviae* by PCR with primers designed from a highly conserved region among various PHA synthases. The amplified fragment was then used as a specific probe for identification and isolation of the PHA biosynthesis genes of *A. caviae*. These PCR primers are expected to be useful for cloning of PHA synthase genes from other bacteria.

The nucleotide sequence indicated that $phaC_{Ac}$ was clustered with four ORFs (ORF1, -3, -4, and -5) and one putative promoter region in a 5.0-kbp genomic fragment. The deduced amino acid sequence of *A. caviae* PHA synthase shows 42.7% identity with the synthase of *A. eutrophus* (specific for short-chain-length 3HA), which is higher than its 32.6 to 35.2% identity with those of pseudomonads (specific for medium- and

TABLE 2. Accumulation of PHA in recombinant strains harboring PHA biosynthesis genes of *A. caviae*[a]

Strain	Plasmid	Carbon source	PHA content (wt%)	Composition (mol%)	
				3HB	3HHx
A. eutrophus PHB⁻4	pJRD215	Fructose	0		
		Hexanoate	0		
		Octanoate	0		
	pJRDEE50	Fructose	7	100	0
		Hexanoate	Trace		
		Octanoate	6	96	4
	pJRDEE32	Fructose	53	100	0
		Hexanoate	6	83	17
		Octanoate	33	78	22
P. putida GPp104	pJRD215	Gluconate	0		
		Hexanoate	0		
		Octanoate	0		
	pJRDEE50	Gluconate	4	71	29
		Hexanoate	42	61	39
		Octanoate	41	71	29
	pJRDEE32	Gluconate	4	71	29
		Hexanoate	38	60	40
		Octanoate	48	69	31

[a] Cells were cultivated in a nitrogen-limited mineral salt medium containing fructose (0.5% wt/vol), the sodium salts of gluconate (1.5% wt/vol), and octanoate or hexanoate (0.1% wt/vol × 5 for *A. eutrophus* strains or 0.5% wt/vol for *P. putida* strains) as a sole carbon source for 72 h at 30°C.

TABLE 3. Accumulation of PHA in recombinant strains of *A. eutrophus* PHB⁻4 harboring deleted clones of PHA biosynthesis genes of *A. caviae*

Plasmid (relevant markers)	Carbon source	One-step cultivation[a]			Two-step cultivation[b]		
		PHA content (wt%)	Composition (mol%)		PHA content (wt%)	Composition (mol%)	
			3HB	3HH		3HB	3HHx
pJRDEE32 (*phaC*$_{Ac}$, ORF1, ORF3)	Fructose	53	100	0			
	Hexanoate	6	83	17	4	76	24
	Octanoate	33	78	22	6	85	15
pJRDEE32d1 (*phaC*$_{Ac}$, ORF3)	Fructose	66	100	0			
	Hexanoate	78	84	16	25	77	23
	Octanoate	92	87	13	53	85	14
pJRDEE32d3 (*phaC*$_{Ac}$, ORF1)	Fructose	66	100	0			
	Hexanoate	44	75	25	19	53	47
	Octanoate	92	88	12	23	87	13
pJRDEE32d13 (*phaC*$_{Ac}$)	Fructose	73	100	0			
	Hexanoate	72	72	28	26	50	50
	Octanoate	96	85	15	50	80	20

[a] Cells were cultivated in a nitrogen-limited mineral salt medium containing fructose (0.5% wt/vol) and sodium hexanoate or sodium octanoate (0.1% wt/vol × 5) as a sole carbon source for 72 h at 30°C.

[b] Cells were grown in a nutrient-rich medium for 12 h at 30°C, transferred into a nitrogen-free mineral salt medium containing sodium hexanoate or sodium octanoate (0.25% wt/vol × 2) as a carbon source, and incubated for 48 h at 30°C.

long-chain-length 3HA). Although *R. ruber* synthesizes copolyesters of C_4 to C_6 units similar to those of *A. caviae* (12), the PHA synthase of *R. ruber* has closer identity to the synthases of *Pseudomonas aeruginosa* than to those of *A. eutrophus* and *A. caviae*. It is difficult to predict the substrate specificity of PHA synthases on the basis of their primary structures.

Heterologous expression of *phaC*$_{Ac}$ in the PHA-negative mutants PHB⁻4 of *A. eutrophus* and GPp104 of *P. putida* resulted in the accumulation of P(3HB-*co*-3HHx) copolyesters from hexanoate or octanoate and of P(3HB-*co*-3-hydroxyvalerate) from pentanoate (data not shown). A 3-hydroxyoctanoate unit was never detected, even in the copolyesters produced by transconjugants of GPp104 from octanoate. These results indicate that the *A. caviae* PHA synthase is active toward C_4-to-C_6 3HA-CoA and that the composition of the copolyester produced by *A. caviae* FA440 reflects the substrate specificity of the PHA synthase. The acceptance of 3HHx-CoA as a substrate is a significant difference between the *A. caviae* PHA synthase and other synthases, being specific for short-chain-length C_3-to-C_5 3HA-CoA only.

E. coli strains expressing the ORF3 gene of *A. caviae* showed (*R*)-specific enoyl-CoA hydratase activity, suggesting that the translated product of ORF3 functions as an enzyme in a monomer-supplying pathway for PHA-biosynthesis. Figure 4 shows a proposed PHA biosynthesis pathway in *A. caviae*. Acyl-CoA derived from alkanoic acids or oils is degraded via cyclic β-oxidation, resulting in the formation of enoyl-CoA intermediates of different chain lengths. These intermediates may be converted to (*R*)-3HA-CoA by the (*R*)-specific enoyl-CoA hydratase encoded by ORF3, and the resultant (*R*)-3HA-CoA of 4 to 6 carbon atoms may be incorporated into a growing polyester chain by the function of PHA synthase. Hence, P(3HB-*co*-3HHx) is synthesized from alkanoic acids of even carbon numbers or from oils. Small amounts (5 mol% ± 2 mol%) of

TABLE 4. PHA synthase activity and PHA accumulation in recombinant strains of *A. eutrophus* at the early stationary growth phase[a]

Strain	Plasmid (relevant markers)	Carbon source	PHA synthase[b] (U/g of protein)	PHA content (wt%)	Composition (mol%)	
					3HB	3HHx
H16	pJRD215	Fructose	138	60	100	0
		Octanoate	61	67	100	0
PHB⁻4	pJRD215	Fructose	5	0		
		Octanoate	3	0		
PHB⁻4	pJRDEE32 (*phaC*$_{Ac}$, ORF1, ORF3)	Fructose	145	25	100	0
		Octanoate	20	12	61	39
PHB⁻4	pJRDEE32d1 (*phaC*$_{Ac}$, ORF3)	Fructose	33	49	100	0
		Octanoate	13	51	88	12
PHB⁻4	pJRDEE32d3 (*phaC*$_{Ac}$, ORF1)	Fructose	110	28	100	0
		Octanoate	114	37	85	15
PHB⁻4	pJRDEE32d13 (*phaC*$_{Ac}$)	Fructose	425	69	100	0
		Octanoate	770	69	85	25

[a] Cells were cultivated in a nitrogen-limited mineral salt medium containing fructose (0.5% wt/vol) or sodium octanoate (0.1% wt/vol × 3) as a sole carbon source for 30 h at 30°C.

[b] PHA synthesis activity for (*R*)-3HB-CoA.

TABLE 5. Enoyl-CoA hydratase activity in recombinant strains of *E. coli* DH5α[a]

Plasmid	Relevant markers	Enoyl-CoA hydratase[b] (U/g of protein)
pUC18		39
pEE32	$phaC_{Ac}$, ORF1, ORF3	617
pEE32d1	$phaC_{Ac}$, ORF3	323
pEE32d3	$phaC_{Ac}$, ORF1	31
pEE32d13	$phaC_{Ac}$	46

[a] Cells were cultivated in a Luria-Bertani medium for 24 h at 37°C.
[b] Hydration activity for crotonyl-CoA.

a 3HB unit were reported to be incorporated into a copolyester with a 3-hydroxyvalerate unit (95 mol% ± 2 mol%) by *A. caviae* from alkanoic acids of odd carbon numbers (7). In *A. caviae*, there are low activities of β-ketothiolase (0.09 U/mg of protein) and NADH-acetoacetyl-CoA dehydrogenase (0.06 U/mg of protein), but no activity of NADPH-acetoacetyl-CoA reductase was detected. The small amount of a 3HB unit in the copolyesters may be supplied from two acetyl-CoA molecules via four-step reactions catalyzed by β-ketothiolase, NADH-acetoacetyl-CoA dehydrogenase, crotonase [(*S*)-specific enoyl-CoA hydratase], and (*R*)-specific enoyl-CoA hydratase (Fig. 4), which is similar to the pathway in *R. rubrum* (23). The deletion of ORF3 from the EE32 fragment resulted in an enrichment of the 3HHx fraction in P(3HB-*co*-3HHx) produced by the recombinant strains of *A. eutrophus* PHB⁻4 (Table 3). This result may suggest the major contribution of the ORF3 product in supplying a 3HB unit; that is, the (*R*)-specific enoyl-CoA hydratase encoded by ORF3 may be more specific for the hydration of crotonyl-CoA than for that of medium-chain-length enoyl-CoA.

The transconjugant of *A. eutrophus* PHB⁻4 harboring pJRDEE50 accumulated a smaller amount of polyesters than that harboring pJRDEE32, while there was little difference in the PHA content and composition between transconjugants of

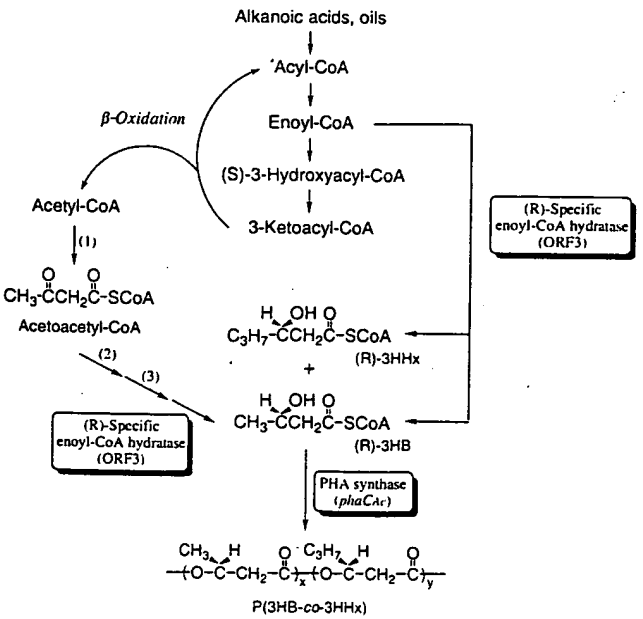

FIG. 4. Proposed pathway of P(3HB-*co*-3HHx)-biosynthesis in *A. caviae* from alkanoic acids or oils. (1) β-Ketothiolase; (2) NADH-acetoacetyl-CoA dehydrogenase; (3) crotonase [(*S*)-specific enoyl-CoA hydratase].

P. putida GPp104 harboring pJRDEE50 and those harboring pJRDEE32. The functions of ORF4 and ORF5 in PHA biosynthesis are still unknown. The deletion of ORF1 and/or ORF3 from the EE32 fragment drastically increased the P(3HB-*co*-3HHx) content in the cells of recombinant PHB⁻4 when hexanoate or octanoate was fed as a sole carbon source. Here, it is noted that a PHA content of 96 wt% was achieved in the strain harboring pJRDEE32d13 from octanoate. The coexpression of ORF1 and ORF3 with $phaC_{Ac}$ seems to lead to a decrease in intracellular PHA content. Enzymatic analysis revealed that the deletion of the surrounding genes also affected the activity of PHA synthase. However, the PHA content accumulated in cells was not correlated to the level of PHA synthase activity (Table 4). For example, the PHA content in octanoate-grown cells harboring pJRDEE32d13 was only slightly higher than that of cells harboring pJRDEE32d1 at both the early stationary (30 h) and late stationary (72 h) phases, even though the former strain showed 60-fold higher synthase activity than the latter. In addition, the effects of the deletion of the ORF3 gene on the activity of PHA synthase and on the cellular content of PHA cannot be explained by the catalytic function of the translated product, (*R*)-specific enoyl-CoA hydratase. Further studies, including the investigation of transcriptional efficiency of each transconjugant, will be necessary to elucidate the characters and functions of these ORFs.

Liebergesell et al. (20) have reported that PHA biosynthesis genes of the anoxygenic phototrophic bacteria *C. vinosum*, *Rhodobacter sphaeroides*, and *T. pfennigii* conferred the ability to synthesize P(3HB-*co*-3HHx) from octanoate on *P. putida* GPp104. It is interesting that the PHA synthases of *C. vinosum* and *T. pfennigii* exhibited similar specificity to that of *A. caviae* in spite of their quite different structures. They have also reported that P(3HB) homopolymer was accumulated in the octanoate-grown cells of *A. eutrophus* PHB⁻4 harboring the genes of *T. pfennigii*, in contrast to the production of PHA containing nearly a half-molar ratio of the 3HHx fraction by the GPp104 strain expressing the same genes. In this study, the transconjugant of PHB⁻4 harboring pJRDEE32d13, expressing only $phaC_{Ac}$ as a foreign gene, efficiently accumulated P(3HB-*co*-3HHx) from hexanoate or octanoate, and the 3HHx fraction in the copolyester reached 50 mol% in the recombinant cells from hexanoate by two-step cultivation. Apparently, *A. eutrophus* cells have the ability to supply the (*R*)-3HHx-CoA thioester intermediate from these carboxylic acids. The P(3HB) accumulation from octanoate in recombinant PHB⁻4 strains described by Liebergesell et al. may be caused by the effects of additional genes in the cloned fragment which were introduced into the host together with the PHA synthase gene.

In conclusion, this study not only gives information on PHA biosynthesis genes of *A. caviae* but also demonstrates the usefulness of the recombination techniques for an efficient production of copolyesters consisting of short- and medium-chain-length 3HA-CoA. Further studies will be done to clarify the mechanism of synthesis and accumulation of PHA in *A. caviae* and in recombinant strains harboring the PHA biosynthesis genes of *A. caviae*.

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REFERENCES

1. **Allen, L. N., and R. S. Hanson.** 1985. Construction of broad-host-range cosmid cloning vectors: identification of genes necessary for growth of *Methy-*

lobacterium organophilum on methanol. J. Bacteriol. 161:955–962.
2. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–472.
3. **Brandl, H., E. J. Knee, R. C. Fuller, R. A. Gross, and R. W. Renz.** 1989. Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly(β-hydroxyalkanoates): potential source for biodegradable polyesters. Int. J. Biol. Macromol. 11:49–55.
4. **Davison, J., M. Heusterspreute, N. Chevalier, V. Ha-Thi, and F. Brunel.** 1987. Vectors with restriction site banks. pJRD215, a wide-host-range cosmid vector with multiple cloning sites. Gene 51:275–280.
5. **Deng, W. P., and J. A. Nickoloff.** 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. 200:81–88.
6. **Doi, Y.** 1990. Microbial polyesters. VCH Publishers, New York, N.Y.
7. **Doi, Y., S. Kitamura, and H. Abe.** 1995. Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules 28:4822–4828.
8. **Friedrich, B., C. Hogrefe, and H. G. Schlegel.** 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. 147:198–205.
9. **Gerngross, T. U., K. D. Snell, O. P. Peoples, A. J. Sinskey, E. Csuhai, S. Masamune, and J. Stubbe.** 1994. Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*. Evidence for a required posttranslational modification for catalytic activity. Biochemistry 33:9311–9320.
10. **Hawrey, D. K., and W. R. McClure.** 1983. Composition and analysis of *Escherichia coli* promoter sequences. Nucleic Acids Res. 11:2237–2255.
11. **Haywood, G. W., A. J. Anderson, and E. A. Dawes.** 1989. The importance of PHB-synthase substrate specificity in polyhydroxyalkanoate synthesis by *Alcaligenes eutrophus*. FEMS Microbiol. Lett. 57:1–6.
12. **Haywood, G. W., A. J. Anderson, G. A. Williams, E. A. Dawes, and D. F. Ewing.** 1991. Accumulation of a poly(hydroxyalkanoate) copolymer containing primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. NICMB 40126. Int. J. Biol. Macromol. 13:83–87.
13. **Hiltunen, J. K., B. Wenzel, A. Beyer, R. Erdmann, A. Fosså, and W. H. Kunau.** 1992. Peroxisomal multifunctional β-oxidation protein of *Saccharomyces cerevisiae*. J. Biol. Chem. 267:6646–6653.
14. **Huisman, G. W., E. W. Wonink, R. Meima, B. Kazemier, P. Terpstra, and B. Witholt.** 1991. Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. J. Biol. Chem. 266:2191–2198.
15. **Hustede, E., and A. Steinbüchel.** 1993. Characterization of the polyhydroxyalkanoate synthase gene locus of *Rhodobacter sphaeroides*. Biotechnol. Lett. 15:709–714.
16. **Kato, M., H. J. Bao, C. K. Kang, T. Fukui, and Y. Doi.** 1996. Production of a novel copolyester of 3-hydroxybutyric acids and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars. Appl. Microbiol. Biotechnol. 45:363–370.
17. **Kawaguchi, Y., and Y. Doi.** 1992. Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*. Macromolecules 25:2324–2329.
18. **Lee, S. Y.** 1996. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. 49:1–14.
19. **Liebergesell, M., E. Hustede, A. Timm, A. Steinbüchel, R. C. Fuller, R. W. Lenz, and H. G. Schlegel.** 1991. Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria. Arch. Microbiol. 155:415–421.
20. **Liebergesell, M., F. Mayer, and A. Steinbüchel.** 1993. Analysis of polyhydroxyalkanoic acid-biosynthetic genes of anoxygenic phototrophic bacteria reveals synthesis of a polyester exhibiting an unusual composition. Appl. Microbiol. Biotechnol. 40:292–300.
21. **Liebergesell, M., and A. Steinbüchel.** 1992. Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in *Chromatium vinosum* strain D. Eur. J. Biochem. 209:135–150.
22. **Liebergesell, M., and A. Steinbüchel.** 1993. Cloning and molecular analysis of the poly(3-hydroxybutyric acid) biosynthetic genes of *Thiocystis violacea*. Appl. Microbiol. Biotechnol. 38:493–501.
23. **Moskowitz, G. J., and J. M. Merrick.** 1969. Metabolism of poly-β-hydroxybutyrate. Enzymatic synthesis of D-(-)-β-hydroxybutyryl coenzyme A by an enoyl hydrase from *Rhodospirillum rubrum*. Biochemistry 8:2748–2755.
24. **Mullany, P., C. L. Clayton, M. J. Pallen, R. Slone, A. al-Saleh, and S. Tabaqchali.** 1994. Genes encoding homologues of three consecutive enzymes in the butyrate/butanol-producing pathway of *Clostridium acetobutylicum* are clustered on the *Clostridium difficile* chromosome. FEMS Microbiol. Lett. 124:61–67.
25. **Müller, H. M., and D. Seebach.** 1993. Poly(hydroxyalkanoates): a fifth class of physiologically important organic biopolymers? Angew. Chem. Int. Ed. Engl. 32:477–502.
26. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. 264:15298–15303.
27. **Pieper, U., and A. Steinbüchel.** 1992. Identification, cloning and sequence analysis of the poly(3-hydroxyalkanoic acid) synthase gene of the gram-positive bacterium *Rhodococcus ruber*. FEMS Microbiol. Lett. 96:73–80.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Schembri, M. A., R. C. Bayly, and K. Davies.** 1994. Cloning and analysis of the polyhydroxyalkanoic acid synthase gene from *Acinetobacter* sp. Evidence that the gene is both plasmid and chromosomally located. FEMS Microbiol. Lett. 118:145–152.
30. **Schlegel, H. G., R. Lafferty, and I. Krauss.** 1970. The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch. Mikrobiol. 71:283–294.
31. **Schubert, P., A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* poly-β-hydroxybutyrate synthetic pathway and synthesis of PHB in *Escherichia coli*. J. Bacteriol. 170:5837–5847.
32. **Shimamura, E., K. Kasuya, G. Kobayashi, T. Shiotani, Y. Shima, and Y. Doi.** 1994. Physical properties and biodegradability of microbial poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules 27:878–880.
33. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering. Transposon mutagenesis in gram negative bacteria. Bio/Technology 1:784–791.
34. **Slater, S. C., W. H. Voige, and D. E. Dennis.** 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J. Bacteriol. 170:4431–4436.
35. **Steinbüchel, A.** 1996. PHB and other polyhydroxyalkanoic acids. *In* H. J. Rehm and G. Reed (ed.), Biotechnology. VCH Publishers, Weinheim, Germany.
36. **Steinbüchel, A., E. Hustede, M. Liebergesell, U. Pieper, A. Timm, and H. Valentin.** 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol. Rev. 103:217–230.
37. **Steinbüchel, A., and H. E. Valentin.** 1995. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. 128:219–228.
38. **Steinbüchel, A., and S. Wiese.** 1992. A *Pseudomonas* strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 37:691–697.
39. **Timm, A., and A. Steinbüchel.** 1992. Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of *Pseudomonas aeruginosa* PAO1. Eur. J. Biochem. 209:15–30.
40. **Valentin, H. E., E. Y. Lee, C. Y. Choi, and A. Steinbüchel.** 1994. Identification of 4-hydroxyhexanoic acid as a new constituent of biosynthetic polyhydroxyalkanoic acids from bacteria. Appl. Microbiol. Biotechnol. 40:710–716.
41. **Valentin, H. E., A. Schönebaum, and A. Steinbüchel.** 1996. Identification of 5-hydroxyhexanoic acid, 4-hydroxyheptanoic acid and 4-hydroxyoctanoic acid as new constituents of bacterial polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 46:261–267.
42. **Valentin, H. E., and A. Steinbüchel.** 1993. Cloning and characterization of the *Methylobacterium extorquens* polyhydroxyalkanoic-acid-synthase structural gene. Appl. Microbiol. Biotechnol. 39:309–317.
43. **Valentin, H. E., and A. Steinbüchel.** 1994. Application of enzymatically synthesized short-chain-length hydroxy fatty acid coenzyme A thioesters for assay of polyhydroxyalkanoic acid synthases. Appl. Microbiol. Biotechnol. 40:699–709.